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(54) Title: <b>METHODS AND COMPOSITIONS FOR DECREASING MITOCHONDRIAL OVERPRODUCTION OF REACTIVE OXYGEN SPECIES IN CELLS</b>		
(57) Abstract  <p>The subject invention provides means of identifying those patients most in need of treatment to prevent the development or progression of ROS mediated complications of diabetes or hyperglycemia, as well as methods of treating a patient in need thereof. Pharmacologic treatment is by providing the patient with an agent identified as reducing levels of ROS or ROS level correlated indicators such as levels of LPs, AGEs, NFκB, PKC, or aldose reductase activation in insulin-independent cells grown under high glucose conditions. All agents that are identified as efficacious can be employed as pharmacotherapeutic agents if they are otherwise pharmacologically appropriate. Hyperglycemia-induced ROS formation in insulin-independent cells such as endothelial cells is prevented by uncouplers of mitochondrial electron transport, inhibitors of Complex II and superoxide dismutase/catalase mimetics. Inhibitors of Complex II and uncouplers also inhibit hyperglycemia-induced lipid peroxidation, PKC activation, NFκB activation, aldose reductase activity and intracellular glycoxidation product formation. Also provided are methods of gene therapy to reduce ROS and ROS related levels of indicators such as LPs, AGEs, NFκB activation and PKC activation in insulin-independent cells. Gene therapy vector constructs, including viral vector constructs, are introduced into the cells of a patient in need thereof. Such constructs code for a peptide that uncouples oxidative phosphorylation. Alternatively the invention describes a method for provision of a replacement functional or normal peptide for a defective mitochondrial gene product which is identified by the method described above as associated clinically with the development or progression of diabetic complications. In addition to providing a method for mitochondrial gene therapy for patients in need thereof, the invention provides for a method of selecting non-mutated mitochondria by transfecting a cell with peptide-nucleic acids having sequences complementary to mitochondrial DNA that contain a deletion breakpoint or single base mutation as compared to wild-type mitochondrial DNA so that replication of the mitochondrial DNA is blocked and mitochondria with DNA mutations are eliminated.</p>		

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## METHODS AND COMPOSITIONS FOR DECREASING MITOCHONDRIAL OVERPRODUCTION OF REACTIVE OXYGEN SPECIES IN CELLS

### INTRODUCTION

#### Field of the Invention

The field of the invention is related to methods and compositions for decreasing accumulation and/or increasing degradation of reactive oxygen species (ROS) that damage cells, such as those involved in vascular and/or neurological disease and/or dysfunction, with intracellular hyperglycemia as a means of preventing and/or treating diabetic and hyperglycemic tissue damage or organ damage as well as age-related and other diseases in which tissues are damaged due to intracellular ROS production by mitochondria. The invention is exemplified by the use of carbonyl cyanide *m*-chlorophenylhydrazone, theonyltrifluoroacetone and Mn (111) tetrakis (benzoic acid) porphyrin to decrease ROS generation and consequences, such as PKC activation, in insulin-independent cells in the presence of high glucose, the use of theonyltrifluoroacetone and Mn (111) tetrakis (benzoic acid) porphyrin to decrease ROS in liver cells and the use of carbonyl cyanide *m*-chlorophenylhydrazone and theonyltrifluoroacetone to inhibit leptin induced ROS in vascular cells.

#### Background

There are an estimated 14 million diabetics in the USA, and a similar number in Europe. Diabetic complications are divided into those affecting the small blood vessels in the retina, kidney, and nerve, as well as nerve cells (microvascular complications), and those affecting the large blood vessels supplying the heart, brain, and lower limbs (macrovascular complications). Atherosclerosis is several-fold more frequent in diabetic than non-diabetic subjects. The lesions are similar to those in the general population, but are more severe and widespread: coronary artery disease in particular may progress more rapidly. Diabetes or impaired glucose-tolerance itself confer 75-90% of the excess risk of coronary disease. However, atherosclerosis correlates only weakly with the duration or severity of glucose intolerance, suggesting that hyperglycemia per se may be primarily responsible. In fact, asymptomatic subjects with impaired glucose tolerance or borderline fasting hyperglycemia

also have an increased risk of cardiovascular disease. Insulin resistance and/or high insulin levels may be atherogenic, but the possible atherogenic role of insulin in humans remains controversial (Gray, RP, and Yudkin, J.S., Cardiovascular disease in diabetes mellitus, in Textbook of Diabetes, Eds. JC Pickup and G. Williams, 2<sup>nd</sup> edition, Blackwell Science Ltd. Oxford 1997).

Obesity also is associated with an increased risk of cardiovascular disease. Leptin, the product of the *ob* gene, is a plasma protein secreted by adipocytes and is involved in the control of body weight, mainly through its hypothalamic effects (*Science* 269:540-543, 1995; *Science* 269:543-546, 1995; *Science* 269:546-549). Serum leptin concentrations are elevated in insulin-resistant non-diabetic first-degree relatives of patients with Type II diabetes, as well as in Type II diabetics, suggesting that insulin resistance causes elevated leptin levels. Leptin levels are also elevated in Type I diabetics, independent of body mass index (Nyholm, B., *et al.*, *European J. of Endocrinology* 136:173-179, 1997; Kamoda, T., *et al.*, *Clinical Endocrinology* 49: 385-389, 1998; Rudberg, S., and Persson, B. *Horm. Res* 50:297-302, 1998).

Microvascular complications related to diabetes are the leading cause of new blindness in people 20-74 years old, and account for 35% of all new cases of end-stage renal disease. Over 60% of diabetics are affected by neuropathy, which includes distal symmetrical sensory polyneuropathy, mononeuropathies, and a variety of autonomic neuropathies causing erectile dysfunction, urinary incontinence, gastroparesis and nocturnal diarrhea. Diabetes accounts for 50% of all non-traumatic amputations in the USA, primarily as a result of diabetic macrovascular disease, and diabetics have a death rate from coronary artery disease that is 2.5 times that of non-diabetics.

Hyperglycemia is believed to initiate and accelerate progression of diabetic microvascular complications based upon the fact that target tissues of complications tend to be those that do not require insulin for glucose transport into cells and/or which do not down-regulate glucose transport with hyperglycemia, thus putting mechanistic focus on the consequences of intracellular hyperglycemia. Various intracellular mechanisms have been proposed to explain the damage that occurs and used as a basis for treatment of hyperglycemia induced complications.

The first focuses on the sorbitol/polyol pathway: increased glucose is metabolized to sorbitol by the enzyme aldose reductase and NADPH. Sorbitol is then oxidized to fructose by

the enzyme sorbitol dehydrogenase and NAD. Increased operation of this pathway is associated with a number of functional abnormalities in diabetic cells and vessels, which are attenuated by aldose reductase inhibitors. Experimental treatments using aldose reductase inhibitors pose the problem that one of the functions of aldose reductase is to detoxify 4-OH-nonenal, a lipid peroxidation product that can damage and/or kill cells.

The second focuses on the altered redox state (decreased NAD/NADH) in the cell: increased glucose results in a decreased NAD/NADH ratio, possibly due to the second reaction of the sorbitol pathway. Vascular abnormalities associated with this are normalized by pyruvate, which produces NAD by the action of lactate dehydrogenase. However, pyruvate is a substrate for mitochondria and this treatment therefore has the disadvantage that it would generate free radicals in the mitochondria.

In the third proposed mechanism, the focus is on diacylglycerol (DAG)/protein kinase C (PKC) activation: increased glucose results in the production of DAG and the subsequent activation of various PKC isoforms by DAG. Various vascular abnormalities are corrected by a specific PKC isoform inhibitor. The disadvantages associated with the use of PKC inhibitors, even those that are theoretically specific, is that it is unlikely that the inhibitor is completely specific and/or it could inhibit essential PKC functions.

In the fourth, nonenzymatic glycation/advanced glycation endproducts (AGEs) is the focus: glucose and glucose metabolites covalently attach to amino groups of proteins and form a variety of complex AGE adducts, including cross-links. Aminoguanidine prevents the formation of AGEs, in part by trapping reactive sugar-derived intermediates, and prevents most diabetic microvascular complications in animal models. Aminoguanidine has a number of effects, including inhibiting an inducible enzyme that makes nitric oxide in macrophages to fight infection. This treatment therefore has the disadvantage that it would impair immune function. In addition, aminoguanidine exhibits tissue toxicity.

In the fifth hypothesis, the focus is on oxidative stress/lipid peroxidation: hyperglycemia causes increased intracellular reactive oxygen species and lipid peroxidation. A variety of deleterious consequences of these processes has been documented, including activation of NF $\kappa$ B. A disadvantage of using non-catalytic antioxidants such as Vitamin E, ascorbate and glutathione is their finite ability to titrate continuously produced ROS. ROS scavengers such as superoxide dismutases and catalase, as proteins, have a number of delivery and other shortcomings.

It therefore is of interest to determine whether there is a common link between hyperglycemia and the five mechanisms outlined above as a means of developing methods and compositions for treating and/or preventing diabetic complications.

### RELEVANT LITERATURE

There is no increased extracellular oxidative stress in diabetes (Wells-Knecht *et al*, *J Clin Invest* 100(4):839-846, 1997), but hyperglycemia causes increased intracellular reactive oxygen species (ROS ) (Kannan and Jain, *Hormone & Metabolic Research* 26:322-5, 1994; Habib *et al*, *Metabolism* 43:1442-5, 1994; Jain *et al*, *Molecular and Cellular Biochemistry* 162:11-6, 1996; Giardino *et al*, *J Clin Invest* 97:1422-28, 1996). ROS activate aldose reductase (Grimshaw and Lai, *Arch Biochem Biophys* 327:89-97, 1996; Ou *et al*, *Free Radical Res* 25:337-346, 1996), and cause a decline in the NAD/NADH ratio (Thies and Autor, *Arch Biochem Biophys* 286:353-363, 1991), induce DAG and activate PKC (Taher *et al*, *Archives of Biochem & Biophys* 303:260-6, 1993; Gopalakrishna *et al*, *PNAS* 91:12233-7, 1994; Kuo *et al*, *Biochimica Biophysica Acta* 1268:229-36, 1995; Nakajima and Yukamwa, *International Journal of Radiation Biology* 70:473-80, 1996; Klann *et al*, *J Biol Chem* 273:4516-22, 1998), induce advanced glycation endproduct formation (Giardino *et al*, *J Clin Invest* 97:1422-28, 1996; Fu *et al*, *Diabetes* 43:676-83, 1994; Elgawish *et al*, *J Biol Chem* 271:12964-71, 1996), and induce lipid peroxidation. In addition, ROS activate the pleiotrophic transcription factor NF $\kappa$ B (Piette *et al*, *Biological Chemistry* 378:1237-45, 1997).

Antioxidants (*o*-phenanthraline, thiocctic acid) prevent aldose reductase activation (Ou *et al*, *Free Radical Res* 25:337-346, 1996), normalize altered redox state (SOD/catalase, desferoxamine) (Thies and Autor, *Arch Biochem Biophys* 286:353-363, 1991), inhibit PKC activation (taurine, N-acetylcysteine) (Wolf *et al*, *J Clin Invest* 87:31-38, 1991; Studer *et al*, *Metabolism: Clinical and Experimental* 46:918-25, 1997), prevent AGE formation (deferoxamine, alpha tocopherol, DMSO, penicillamine, catalase) (Giardino *et al*, *J Clin Invest* 97:1422-28, 1996; Fu *et al*, *Diabetes* 43:676-83, 1994; Elgawish *et al*, *J Biol Chem* 271:12964-71, 1996), and prevent lipid peroxidation (deferoxamine, alpha tocopherol, DMSO) (Giardino *et al*, *J Clin Invest* 97:1422-28, 1996; Kaul *et al*, *Mol Cell Biochem* 160: 283-8, 1996). Antioxidants also prevent NF $\kappa$ B activation (Piette *et al*, *Biological Chemistry* 378:1237-45, 1997). Aldose reductase inhibitors (ARIs) also prevent loss of the cellular antioxidant taurine and chelate ROS-forming divalent metal ions (Ou *et al*, *Free Radical Res*

25:337-346, 1996; Malone *et al*, *Pediatric Research* 27:293-6, 1990). The redox-correcting compound pyruvate is also a peroxide/hydroxyl scavenger (O'Donnell-Tormey *et al*, *J Exp Med* 165:500-14, 1987; Lee *et al*, *J Biol Chem* 273:5294-9, 1998). The AGE-inhibitor aminoguanidine prevents hyperglycemia-induced ROS formation *in vitro* and *in vivo* (Giardino *et al*, *Diabetes* 47:1114-1120, 1998).

Superoxide dismutase mimetics have been reported to protect endothelial cells against hydrogen peroxide mediated injury (Day *et al*, *Archives Biochem. Biophys* (1997) 347:256-262) and to inhibit neuronal apoptosis (Patel, *J. Neurochem* (1998) 71:1068-1074). A review of the pharmacological effects of superoxide dismutase mimetics is provided by Doctrow *et al*, *Advances In Pharmacology* (1997) 38:247-269.

Melov *et al*, *Nature Genetics* 18:159-63, 1998 report that in mitochondrial (manganese) SOD knockout mice, dilated cardiomyopathy and early neonatal death were prevented by intraperitoneal injection of MnTBAP. They still developed neurologic problems because MnTBAP does not cross the blood brain barrier.

### SUMMARY OF THE INVENTION

This invention is directed to methods and compositions for preventing and/or treating hyperglycemic or diabetic complications in a patient in need thereof by directly or indirectly decreasing the mitochondrial reactive oxygen species (ROS) generated in insulin-independent cells of the patient, particularly vascular cells and cells comprising peripheral neurons, or by counteracting the effects of ROS-induced mitochondrial DNA mutations. Embodiments of the methods for preventing and/or treating tissue or organ abnormalities associated with diabetes and hyperglycemia include (1) providing the patient with a composition comprising an agent that decreases the level of hyperglycemia induced reactive oxygen species produced in insulin-independent cells, particularly by normal mitochondria; (2) reducing the number of mitochondria with DNA mutations that result from production of reactive oxygen species in insulin-independent cells by transfecting a cell with peptide-nucleic acids having sequences complementary to mitochondrial DNA that contain a deletion breakpoint or single base mutation as compared to wild-type mitochondrial DNA; and (3) decreasing the effects of an abnormal mitochondrial DNA gene product in a patient by introducing into the patient an expression cassette comprising as operatively linked components a transcriptional initiation region, a nucleotide sequence encoding an amino-terminal mitochondrial targeting sequence

and a normal counterpart of the abnormal mitochondrial gene under conditions whereby the normal gene is expressed.

The invention additionally encompasses methods of screening for pharmaceutical agents that can be used for treating diabetic and hyperglycemic patients. The method includes screening for agents that (1) decrease the level of or inhibit the formation of hyperglycemia-induced reactive oxygen species under high glucose conditions, including agents effective in partially uncoupling oxidative phosphorylation from electron transport in mitochondria; (2) increase degradation of reactive oxygen species (for example, superoxide dismutase/catalase mimetics); (3) inhibit mitochondrial electron transport complexes; or (4) inhibit binding and activation of hexokinase isoforms to or by the mitochondrial membrane. The pharmaceutical agents identified find use in treating symptoms associated with diabetes and hyperglycemia in a patient in need thereof or other diseases in which tissue damage is related to increased ROS.

In another aspect of the invention, a patient's risk for development or progression of diabetic or hyperglycemic complications is determined by evaluating whether there is an increase in either (1) the levels of reactive oxygen species generated in fusion cells obtained by fusing non-nucleated or enucleated insulin-independent cells from a patient with nucleus donor cells lacking mitochondrial DNA, as compared to levels of reactive oxygen species in control cells or (2) the presence of risk-associated homoplasmic polymorphisms in mitochondrial DNA in cells obtained from the patient as compared to a risk index for complications obtained by correlating the mitochondrial DNA sequences of homoplasmic polymorphisms in insulin-independent cells obtained from individual members of a group of diabetics with development or progression of diabetic complications.

The invention finds use in the treatment and/or prevention of hyperglycemic or diabetic complications as well as symptoms of other diseases in which tissue is damaged due to elevated intracellular ROS production by mitochondria by reducing levels of ROS either by preventing production or increasing degradation. These diseases include Alzheimer's Disease, Parkinson's Disease, and other age-related, tissue-degenerative diseases, as well as the arterogenic effects of elevated leptin, for example in patients with impaired glucose tolerance and obese non-diabetic patients.



### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the effect of specific metabolic inhibitors on hyperglycemia-induced reactive oxygen species (ROS) formation in bovine aortic endothelial cells. Cells were incubated (n=24) in 5mM glucose alone, and in 30mM glucose, alone, for 24 hours and with either aminooxyacetate (AOAC), 4-hydroxycyanocinnamic acid (4-OHCA), amytal, thenoyltrifluoroacetone (TTFA), or carbonyl cyanide m-chlorophenylhydrazone (CCCP), and ROS were quantitated. # = P < 0.001 compared to cells incubated in 5mM glucose. # = P < 0.001 compared to cells incubated in 30mM glucose.

Figure 2 shows the effect of different mitochondrial blockades on hyperglycemia-induced cellular lipid peroxidation in BAE cells determined as thiobarbituric acid reactive substances (TBARS). BAE cells were incubated for 168 hours in media containing the following: (i) 5mM glucose (low glucose); (ii) 30mM glucose (high glucose); (iii) 30mM glucose plus 2mM azide; (iv) 30mM glucose plus 0.5  $\mu$ M CCCP; (v) 30mM glucose plus 10  $\mu$ M TTFA. The results are expressed as mean  $\pm$  SE of three experiments.

Figure 3 shows the effect of different mitochondrial blockades on hyperglycemia-induced activation of NF $\kappa$ B in BAE cells as measured by an electrophoretic mobility shift assay. In Figure 3A are shown the results of incubating BAE cells for 2 hours in media containing the following: (i) 5mM glucose (low glucose); (ii) 30mM glucose (high glucose); (iii) 30mM glucose plus 2mM azide; (iv) 30mM glucose plus 0.5  $\mu$ M CCCP; (v) 30mM glucose plus 10  $\mu$ M TTFA; (vi) 30mM glucose plus 0.5  $\mu$ M oligomycin. The results are expressed as mean  $\pm$  SE of three experiments. Figure 3B shows the effect of electron transport Complex II inhibition or oxidative phosphorylation uncoupling on hyperglycemia-induced activation of NF $\kappa$ B. Cells were incubated 7 days in 5mM glucose alone, and in 30mM glucose, either alone, or with either thenoyltrifluoroacetone (TTFA) or carbonyl cyanide m-chlorophenylhydrazone (CCCP), and an electrophoretic mobility shift assay was performed.

Figure 4 shows the effect of different mitochondrial blockades on hyperglycemia-induced formation of methyglyoxal-derived advanced glycation endproducts (AGEs) in BAE cells determined by scanning densitometry of IH7G5 antibody immunoblots. BAE cells were incubated for 168 hours in media containing the following: (i) 5mM glucose (low glucose); (ii) 30mM glucose (high glucose); (iii) 30mM glucose plus 2mM azide; (iv) 30mM glucose plus 0.5  $\mu$ M CCCP; (v) 30mM glucose plus 10  $\mu$ M TTFA. The results are expressed as mean  $\pm$  SE of three experiments.

Figure 5 shows the effect of different mitochondrial blockades on hyperglycemia-induced formation of glyoxal and/or fructosyllysine-derived advanced glycation endproducts (AGEs) in BAE cells determined by scanning densitometry of 6D12 antibody immunoblots. BAE cells were incubated for 168 hours in media containing the following: (i) 5mM glucose (low glucose); (ii) 30mM glucose (high glucose); (iii) 30mM glucose plus 2mM azide; (iv) 30mM glucose plus 0.5  $\mu$ M CCCP; (v) 30mM glucose plus 10  $\mu$ M TTFA. The results are expressed as mean  $\pm$  SE of three experiments.

Figure 6 shows the effect of different mitochondrial blockades on PKC activity in BAE cells. BAE cells were incubated for 5 days in media containing the following: (i) 5mM glucose (low glucose); (ii) 30mM glucose (high glucose); (iii) 30mM glucose plus 10  $\mu$ M TTFA; (iv) 30mM glucose plus 0.5  $\mu$ M CCCP. The results are expressed as mean  $\pm$  SE of four experiments. \* $p$  < 0.01 as compared to 5mM glucose.

Figure 7 shows the effect of electron transport Complex II inhibition on hyperglycemia-induced sorbitol accumulation resulting from aldose reductase activity. BAE cells were incubated for 168 hours in either 5mM glucose alone (n=9), or in 30mM glucose (n=5), with or without thenoyltrifluoracetone (TTFA) (n=6), and intracellular sorbitol content was determined. \*= $P$ <0.001 compared to cells incubated in 5mM glucose. #= $P$ <0.001 compared to cells incubated in 30mM glucose.

Figure 8 shows the effect of electron transport Complex II inhibition on hyperglycemia-induced overexpression of the tyrosine kinase vascular endothelial growth factor receptor flk-1 in BAE cells determined by scanning densitometry of immunoblots. BAE cells were incubated for 168 hours in media containing the following: (1) 5mM glucose (low glucose); (2) 30mM glucose (high glucose); (3) 30mM glucose plus 10 $\mu$ M TTFA. The results are expressed as mean  $\pm$  SE of three experiments.

Figure 9 shows the effect of a superoxide dismutase mimetic on hyperglycemia-induced reactive oxygen species production (micromoles/ml). BAE cells were incubated for 24 hours in 5mM glucose alone and in 30mM glucose alone, and with one of the indicated concentrations of Mn (111) tetrakis (benzoic acid) porphyrin (MnTBAP). The results are expressed as mean  $\pm$  SE of 8 experiments.

Figure 10 shows the effect of different mitochondrial blockades on hyperglycemia-induced reactive oxygen species accumulation (micromoles/ml) in cultured rat hepatocytes. Rat hepatocytes were incubated 24 hours in 5mM glucose alone, and in 30mM glucose, alone,

and with either aminooxyacetate (AOAC), 4-hydroxycyanocinnamic acid (4-OHCA), amytal, thenoyltrifluoracetone (TTFA), or carbonyl cyanide m-chlorophenylhydrazone (CCCP) and with one of the indicated concentrations of Mn (111) tetrakis (benzoic acid) porphyrin (MnTBAP) and ROS were quantitated. The results are expressed as mean  $\pm$  SE of 8 experiments. \* =  $P < .001$  as compared to cells incubated in 5mM glucose alone.

Figure 11 shows the effect of different mitochondrial blockades on leptin-induced reactive oxygen species in bovine aortic endothelial cells. The cells were preincubated for an hour in TTFA (10  $\mu$ M) or CCCP (0.5  $\mu$ M) and then incubated for forty five minutes at room temperature with leptin (10 ng/ml). From left to right the solid bars represent control cells; leptin alone; TTFA alone; leptin + TTFA; CCCP alone; and leptin + CCCP. The results are expressed as  $\mu$ moles/ml of ROS.

Figure 12 shows the effect of mitochondrial inhibitors on hyperglycemia-induced increases in glucose-6 phosphatase gene expression. Rat hepatocytes were incubated under standard conditions (Seoane, J., *et al.*, *J. Biol. Chemistry* 272:26972-26977, 1977) with either 5mM glucose, 30mM glucose, or 30 mM glucose plus TTFA, CCCP, or MnTBAP, as described in Example 10. After 24 hours, mRNA was prepared and quantitative RT-PCR performed. Data was normalized for beta actin amplified in the same tubes. The Y-axis shows the densitometric readings which correspond to relative glucose-6 phosphatase mRNA levels. Data represent the mean  $\pm$  SE for 3 experiments.

Figure 13 shows the effect of mitochondrial inhibitors on hyperglycemia-induced increases in hepatic glucose production. Rat hepatocytes were incubated under standard conditions (Seoane, J., *et al.*, *J. Biol Chemistry* 272:26972-26977, 1977) with either 5mM glucose, 30mM glucose, or 30mM glucose plus TTFA, CCCP, or MnTBAP, as described in Example 10. After 24 hours, hepatic glucose output (primarily gluconeogenesis) was determined as described in conditions (Seoane, J., *et al.*, *J. Biol Chemistry* 272:26972-26977, 1977). Briefly, Cells were incubated in the presence of gluconeogenic substrates (5mM alanine, 5mM glycine, 5mM glutamine, 10mM lactate, 1mM pyruvate) in the absence of glucose for 2 hours. Media were collected for glucose determination by the standard glucose oxidase method. Data represent the mean  $\pm$  SE for 9 experiments.

### **BRIEF DESCRIPTION OF THE SPECIFIC EMBODIMENTS**

Methods and compositions are provided for preventing and/or treating hyperglycemic or diabetic complications in a diabetic patients, as well as symptoms of patients with other diseases, in whom tissue is damaged due to elevated intracellular reactive oxygen species (ROS) production by mitochondria by inhibiting mitochondrially derived ROS.

5 Mitochondrial reactive oxygen species generated in insulin-independent hyperglycemic cells of the patient by mitochondria, particularly normal mitochondria can be inhibited either directly or indirectly, or by counteracting the effects of ROS-induced mitochondrial DNA mutations. By hyperglycemic or diabetic complications is intended any of a series of diabetes-induced

10 abnormalities that cause morbidity and premature mortality. These include circulatory abnormalities, neurologic abnormalities and hepatic abnormalities. Circulatory abnormalities in large vessels affect peripheral arteries (causing intermittent claudication, gangrene, and in men, organic impotence), coronary arteries (causing myocardial infarction), and arteries supplying the brain (causing stroke). Circulatory abnormalities in small vessels affect the

15 retina (causing blindness), the kidney (causing renal failure), and the heart (causing heart failure). Neurologic abnormalities affect every part of the nervous system with the possible exception of the brain, causing pain, numbness, autonomic dysfunction, and foot ulcers leading to amputation. Hepatic abnormalities include non-physiologic overproduction of glucose, for example when the liver becomes refractory to the effects of increasing glucose

20 due to exposure to high levels of glucose for an extended period of time, and continues to produce glucose, and production of a pro-atherogenic secreted protein profile. By a diabetic patient is intended a patient with a degree of glucose intolerance sufficient to produce diabetic complications as defined by the American Diabetes Association in *Diabetes Care* (1998) 21: (Supplement 1). By insulin-independent cells is intended cells that either do not require

25 insulin for glucose transport and/or do not down-regulate glucose transport in response to increasing glucose concentrations, i.e., those cells which develop intracellular hyperglycemia in response to high blood glucose, above the normal range, to levels occurring in diabetics.

In the methods, a diabetic patient, or a patient with elevated ROS due to other causes such as increased leptin, can be treated in one of several ways. In the first embodiment, a

30 patient is provided with a composition which comprises an agent that decreases the level of hyperglycemia induced reactive oxygen species in insulin-independent cells. The compositions used include compounds that partially uncouple oxidative phosphorylation from

electron transport in mitochondria, inhibit mitochondrial electron transport Complexes I and/or II, dismutate superoxide and/or hydrogen peroxide, or inhibit binding and activation of hexokinase isoforms to or by the mitochondrial membrane. A second embodiment of treating and/or preventing diabetic and hyperglycemia-related complications is to reduce the number of mitochondria with DNA mutations associated with diabetes that result from production of ROS in insulin-independent cells by transfecting cells of a diabetic patient with peptide nucleic acids having sequences complementary to mitochondrial DNA that contains a deletion breakpoint or single base mutation associated with diabetes as compared to wild-type mitochondrial DNA complexed to mitochondrial targeting peptides. In a third embodiment, the effects of an abnormal mitochondrial DNA gene product in a patient are ameliorated by introducing into the patient an expression cassette comprising as operatively linked components a transcriptional initiation region, a nucleotide sequence encoding an amino-terminal mitochondrial targeting sequence and a normal counterpart of the abnormal mitochondrial gene.

This invention also pertains to methods for identification of a patient's risk for development or progression of diabetic or hyperglycemic complications by comparing the levels of reactive oxygen species in fusion cells obtained by fusing non-nucleated or enucleated insulin-independent cells from a patient with nucleus donor cells lacking mitochondrial DNA, with levels of reactive oxygen species in control fusion cells including cells from patients having hemoplactic polymorphisms in the mitochondrial DNA. The control fusion cells generally are prepared using cells from non-diabetics.

The subject invention has several advantages over existing methods for treating diabetics to prevent or retard diabetic complications. Because the invention centers upon the identification of mitochondrial genetics that predispose the individual patient to diabetic or hyperglycemic complications, it is superior to any prior art method for identification of those at risk for such complications. For the same reason it is a superior method for development of mitochondrial gene therapy and elimination of mitochondria which predispose cells to diabetes instigated tissue or organ damage. The invention identifies the mitochondrial genetics of predisposition to hyperglycemic injury by measuring the chemical species causing the cellular pathology, namely mitochondrial ROS and/or species either caused or correlated to elevated ROS, therefore the invention also is superior to existing methods for identifying

chemical or pharmacologic agents which reduce ROS and therefore have the potential for prophylactics and/or treatment of diabetic complications.

It is a theory of the invention that mitochondrial production of ROS is the central event that leads to tissue or organ damage of cells with intracellular hyperglycemia as well as cells exposed to elevated serum levels of leptin. If this theory is correct, then mechanisms proposed by others and the various dysfunctions related to intracellular damage are not causes but are instead consequences of mitochondrial ROS production. The instant treatments based upon reducing mitochondrial production of ROS therefore are treating the primary abnormality and can be used to prevent not only the known consequences of increased ROS, but also consequences not yet known to be involved with the pathogenesis of organ dysfunction in diabetic patients. The subject invention also offers advantages over the use of antioxidants such as Vitamin E; such treatments generally are not effective because they cannot penetrate into the mitochondrial compartment or because they are used up before they have had the desired effect. If sufficiently large doses of Vitamin E, were given, this would result in bleeding problems because Vitamin E interferes with absorption of Vitamin K. It therefore is more efficient to inhibit mitochondrial production of ROS.

It also is a theory of the invention that hyperglycemia-induced reactive oxygen species in hepatocytes induces increased expression of the enzyme glucose-6-phosphatase and/or products in the liver that increase very low density lipoprotein (VLDL) and low density lipoprotein (LDL), increased oxidized lipoproteins, or decrease high density lipoprotein (HDL). Overproduction of glucose by the liver is the major cause of fasting hyperglycemia in both insulin-dependent and non-insulin-dependent diabetes mellitus. This prolonged hyperglycemia appears to result in a non-physiologic overproduction of glucose by the liver via increased expression of glucose-6-phosphatase (D. Massilon, N. Barzilai, W. Chen *et al.*, *Journal of Biol. Chem.* (1996) 271:9871-4). Since hyperglycemia is the primary initiating factor in the pathogenesis of diabetic complications (J. Skyler, Diabetic Complications: The importance of glucose control. In *Endocrinology and Metabolism Clinics of North America* edited by M. B. Brownlee and G. L. King, Philadelphia: W. B. Saunders 1996), amelioration of this defect would normalize glucose levels and thus prevent diabetic vascular and neurologic complications. In addition, in many diabetic patients, levels of VLDL, LDL and HDL are altered towards an atherogenic profile (Garber, *Medical Clinics of North America* (1998) 82:931-48). The most important atherogenic molecule appears to be an oxidatively-

modified form of low density lipoprotein (Hajjar and Haberland, *J. Biol. Chem.* (1997) 272:22975-8). LDL modified by advanced glycation endproducts may also be more atherogenic (Wang *et al.*, *Proc. Natl. Acad. Sci.* (1998) 95:7643-7; Zhang *et al.*, *Thrombosis and Vascular Biology* (1998) 18:1140-8). Such lipoprotein modification may occur extracellularly, e.g., by extracellular exposure to ROS or AGE-precursors produced by endothelial cells. An unexplored possibility is that lipoprotein modification occurs inside the hepatocytes before the lipoproteins are secreted. In diabetic hepatocytes, hyperglycemia-generated ROS and its consequences could explain the increased amount of atherogenic oxidized and age-modified lipoproteins observed in diabetic patients, as well as atherogenic changes in the production levels of various lipoproteins. Decreasing hyperglycemia-induced ROS in hepatocytes by treating the patient with, for example, a superoxide dismutase mimetic such as TBAP, therefore can play a major role in preventing diabetic complications by restoring the normal responsiveness of the liver to increasing blood glucose.

Agents useful in reducing ROS in insulin-independent cells are identified using a screening assay which uses cultures of any of a variety of mammalian insulin-independent cells, preferably primary cells or cells which have been passaged for only a few passages and retain the intrinsic phenotype of cells, such as endothelial cells, from a particular tissue. For characteristics and methods of screening for appropriate characteristics of endothelial cells in culture, see Craig *et al.*, *Microvascular Research* (1998) 55:65-76. The cells are isolated from tissues which contain insulin-independent cells, including the eye (retinal endothelial cells and pericytes); kidney (glomerulus endothelial cells, epithelial cells, and mesangial cells); artery (arterial endothelial cells and smooth-muscle cells); heart (endothelial cells, and cardiac myocytes); nerve (neurons and Schwann cells); blood (monocytes, macrophages, lymphocytes, and platelets); and hepatocytes. The cell cultures generally are cultured in medium containing either physiologic levels of glucose, 5mM glucose, which is equivalent to a 90 mg/dl blood level, or an amount of glucose which is equivalent to typical blood levels of glucose seen in diabetic patients, generally >5-50mM glucose, preferably 20-40mM glucose, most preferably 30mM glucose, with or without the agent to be tested.

Agents which are candidates for testing include those which inhibit the activity or expression of a molecule, generally an enzyme, in one or more of the pathways for glucose-derived ROS produced by the mitochondria or act as mitochondrial superoxide dismutase mimetics. ROS are produced as a byproduct of electron transport, primarily at the interface

between the flavin dehydrogenases and ubiquinone (CoQ), and at Complex III (Wallace, *Annu.Rev. Biochem.* 61:1175-1212, 1992; Forman and Boveris, *Free Radicals in Biology*, Pryor, WA (ed) vol. V, pp 65-90. Academic Press, 1982; Kwong and Sohal, *Arch. Biochem. Biophys.* (1998) 350:118-126). The principal site of ROS generation differs markedly among  
5 various cell types (Kwong and Sohal, *Arch. Biochem. Biophys.* (1998) 350:118-126).

Electron flow through the mitochondrial electron transport assembly is carried out by 4 inner membrane enzyme Complexes (I-IV), plus cytochrome c and the mobile carrier CoQ. Electrons enter the transport chain from NADH (Complex I) or FADH<sub>2</sub> (Complex II). Electrons from both complexes are transferred to CoQ, and then to Complex III,  
10 Cytochrome c, Complex IV, and finally, molecular oxygen. Electron transfer generates a proton motive force (gradient) that drives ATP synthase (Complex V) (Wallace, *Annu.Rev. Biochem.* 61:1175-1212, 1992; Devlin, *Textbook of Biochemistry*, Wiley-Liss, Inc., New York pp.217-264, 1997). When mitochondria are in cellular respiratory state 4, there is high ATP, high proton motive force (PMF), low electron transport and high leakage of ROS; in  
15 state 3, there is low ATP, discharging PMF, high electron transport and lower leakage of ROS (Skulachev, *Q. Rev. Biophys.* 29:169-2021, 1996; Korshunov *et al.*, *FEBS Letters* 416:15-18, 1997). Examples of agents that can be used to inhibit electron flow generated ROS include, e.g., amytal and rotenone (Complex I); TTFA (Complex II), and partial uncouplers of oxidative phosphorylation, such as CCCP. Inhibitors of Complex I, such as  
20 amytal and rotenone, prevent ROS generation in some systems, for example, the J774.16 macrophage-like cell line (Kiyotaki *et al.*, Oxygen metabolism in cloned macrophage cell lines: glucose dependence of superoxide production, metabolic and spectral analysis. *Journal of Immunology* (1984) 132:857-66), and L929 cells (Schulze-Osthoff *et al.*, *Annu. Rev. Biochem.* (1992) 61:1175-1212).

25 Thenoyltrifluoroacetone (TTFA) decreases the steady-state level of succinate-induced ubisemiquinone radical (Ingledew and Ohnishi, *Biochem. J.* (1977) 164:617-620), but its effect on ROS generation has not been reported previously.

Other partial uncouplers that can be used include protein uncouplers from other cell types. Nucleic acid, particularly cDNA, coding for uncoupling proteins (UCP 1, 2, 3),  
30 expression of which is preferably driven by a glucose responsive promoter element to specifically act during periods of hyperglycemia can be used. Odagiri *et al.*, *J. Biol. Chem.* 271: 1909-15, 1996; Fleury *et al.*, *Nature Genetics* 15:269-72, 1997. Rate limiting electron



acceptors such as CoQ and analogues of CoQ also can be used (Langsjven *et al.*, *PNAS* (1985) 82:4240-4044; Nishikawa *et al.*, *Neurology* (1989) 39:399-403). Electrons channeled via Complex II produce about 4 times more superoxide than those channeled via Complex I (Forman and Boveris, *supra*) and agents that are Complex II inhibitors are therefore preferred as compared to agents that inhibit Complex I. Examples of agents that can inhibit Complex I include amytal and rotenone. Examples of agents that can be used to scavenge superoxide include superoxide/catalase mimetics such as the salen-manganese complexes exemplified by EUK-8 described in Doctrow *et al*, *Advances in Pharmacology* (1997) 38:247-269 and manganese porphyrins described in Day *et al*, *Archives Biochem Biophys* (1997) 347:256-262 and Patel, *J. Neurochem* (1998) 71:1068-1074.

NADH (Complex I) substrate and FADH<sub>2</sub> (Complex II) substrate are generated by mitochondrial TCA-cycle oxidation of cytosolic glycolysis-derived pyruvate (3 moles of NADH to 1 mole of FADH<sub>2</sub>), with additional glucose oxidation-derived NADH equivalent(s) transferred from the cytosol into the mitochondria via the malate (mitochondrial NADH) and/or glycerol phosphate (mitochondrial FADH<sub>2</sub>) shuttles. These shuttles utilize both cytosolic and mitochondrial flavin dehydrogenases (Wallace, *Annu.Rev. Biochem.* 61:1175-1212, 1992; Devlin, *Textbook of Biochemistry*, Wiley-Liss, Inc., New York pp.217-264, 1997). Examples of agents that can be used to inhibit the malate shuttle include aminooxyacetate; and to inhibit the glycerol phosphate shuttle include analogues of glycerol-3-phosphate that are not metabolized.

Increased cytosolic glucose accelerates mitochondrial electron transport by (a) increasing the glucose oxidation rate by inducing mitochondrial binding and activation of hexokinase; (b) increasing cytosolic NADH through glycolysis and other glucose oxidative pathways, and (c) increasing pyruvate formation through glycolysis (Wallace, *supra*; and Devlin, *supra*; Gerbitz *et al.*, *Diabetes* 45:113-126, 1996; Brdiczka and Wallimann, *Mol & Cellular Biochemistry* 133-134:69-83, 1994). Examples of agents that can be used to inhibit mitochondrial binding and activation of hexokinase include glycerol and analogues of glycerol.

Agents of interest are tested by contacting a predetermined quantity of a test compound with the cultured primary or low passage (generally  $\leq 14$  passages) insulin-independent cells and comparing the results of incubating the cells in high glucose with low and high glucose alone. A phenotypic trait in the cells is measured that is related to production of ROS either

directly or indirectly and whether and to what extent the agent of interest can cause the trait to become more similar to that of the cells evaluated in physiologic glucose is determined. The screening assays include the following. Effects on the level of inhibition of hyperglycemia-induced ROS formation in the cell culture system is measured using the fluorescent ROS-detector DCF and FACS as a readout (Giardino *et al.*, *J Clin Invest* 97:1422-28, 1996).

Partial inhibition of Complex II, Complex I, or partial uncoupling action on oxidative phosphorylation, which converts state 4 to a pseudo state 3, is measured by the resultant decrease in ROS production (Skulachev, *Q. Rev. Biophys.* 29:169-2021, 1996; Korshunov *et al.*, *FEBS Letters* 416:15-18, 1997). Complex II-activated antioxidant function (Suno and Nagaoka, *Archives of Gerontology & Geriatrics*. 8(3):291-7, 1989) is assessed by published methods in which lipid peroxidation is measured in the presence and absence of the agent of interest, and the inhibitory effect is blocked by Complex II inhibitors such as TTFA.

Inhibition of the malate shuttle or the glycerol phosphate shuttle causes an inhibition in the ability of mitochondria to utilize glycolysis-derived NADH (Kauppinen *et al.*, *Biochem Biophys Acta* 930:173-8, 1987) and effects on the two shuttles can be evaluated by assessing the activity of cytosolic and mitochondrial malate dehydrogenase and/or glutamate oxaloacetate transaminase (Schiller, *Metabolism: Clinical and Experimental* 28:105-112, 1979) cytosolic and mitochondrial glycerol-3-phosphate dehydrogenase (Smith and Sundaram, *Biochimica et Biophysica Acta*. 884:109-18, 1986; Ishihara *et al.*, *Diabetes* 45:1238-44, 1996). The effect of inhibitors of activation of hexokinase isoforms can be evaluated by separating cytosol and mitochondria by standard techniques after exposure to high glucose and assessing enzyme activity (Adams *et al.*, *Nature Genetics* 15:212-215, 1997).

For evaluating the efficacy of a particular agent *in vivo* for treating diabetes and/or diabetic complications, a number of animal models are used by those of skill in the art and are considered predictive of the efficacy of an agent for treating diabetic patients. For evaluating the efficacy of an agent to reduce glucose in a Type I diabetic, either a rat or a mouse which has been treated with streptozotocin generally is used. For Type II diabetes, a genetically diabetic animal is used, for example a fatty Zucker rat or a db/db mouse. The animals are treated with a test agent, generally up to about 50mg/kg/day and hepatic glucose output, glycosylated hemoglobin or fasting glucose measured at various time points following treatment as compared to control animals. For hepatic glucose output, standard isotope dilution techniques for determining hepatic glucose output can be used or a method employing

administration of labeled gluconeogenic precursor and unlabelled glucose,  
and measuring labelled secreted glucuronate (Hellerstein, *et al. Metabolism: Clinical and Experimental* 46:1390-8, 1997). An animal model of diabetic accelerated atherosclerosis is described in Park *et al. Nature Medicine* 4:1025-31, 1998. apoE knockout mice were made  
5 diabetic with injected streptozotocin, and atherosclerosis was quantitated by morphometry. The diabetic mice apoE null mice had accelerated atherosclerosis compared to the non-diabetic apoE null. The same thing can be done with transgenic mice that overexpress ApoB. A model of diabetic microvascular disease---retinopathy is described in Hammes, *et al., Proc. Natl. Acad. Sci* 89:9364, 1992. Rats were made diabetic by  
10 streptozotocin injection, and the diabetic retinopathy was quantitated by histology (acellular capillaries and microaneurysms) after 75 weeks of diabetes. A model of diabetic microvascular disease---nephropathy is described in Soulis-Liparota, *et al.* Retardation by aminoguanidine of development of albuminuria, mesangial expansion, and tissue fluorescence in streptozotocin-induced diabetic rats is evaluated. After 32 weeks of diabetes, they measured  
15 albumin excretion in the urine, and also glomerular histology (mesangial expansion) by standard morphometric techniques.

For identifying a patient at risk for development or progression of diabetic complications, cultured fusion cells, cybrids, are used. The cybrids are obtained by fusing non-nucleated insulin-independent cells from the patient with nucleus donor cells which lack  
20 mitochondrial DNA (mtDNA). The non-nucleated insulin independent cells can be either circulating or non-circulating cells. Insulin independent cells which naturally do not include a nucleus include platelets. Other non-nucleated insulin independent cells can be obtained by enucleation of the cells using techniques known to those skilled in the art. See for example Laderman *et al., J. Biol. Chem.* 271:15891-7, 1996. Circulating cells for enucleation are  
25 conveniently obtained from a blood sample and include, for example, monocytes, lymphocytes and circulating endothelial cells. Non-circulating cells that are insulin independent can be obtained by standard biopsy techniques from an appropriate tissue. The nucleus donor cells can be any mammalian cell line, preferably a primate cell line, and more preferably a human cell line which lacks mitochondrial DNA. mtDNA of the nucleus donor cells can be removed,  
30 for example, by treatment with ethidium bromide. The risk analysis is carried out by comparing the levels of ROS formed in cybrids produced using the patient cells either under high glucose conditions alone or under both high and normal glucose conditions. The levels

of ROS formed are then compared to the level of ROS in control cybrids obtained by fusing non-nucleated insulin independent cells from non-diabetics. An increased level of ROS in the patient cybrids as compared to the control cybrids identifies a patient at risk for the development or progression of diabetic or hyperglycemic complications. The ROS is measured by fluorescence activated cell sorting. Giardino *et al, supra*.

A second method for predicting the risk of diabetic or hyperglycemic complications in a patient involves preparing a risk index for development of complications. The risk index is obtained by correlating the mtDNA sequences of homoplasmic polymorphisms in insulin independent cells obtained from a plurality of diabetics with developmental progression of diabetic complications in each individual of the plurality of diabetics. The mtDNA sequences of homoplasmic polymorphisms in an individual patient can then be compared with the risk index as a means of predicting the risk of diabetic or hyperglycemic complications. The mtDNA sequences of homoplasmic polymorphisms can be determined according to the method of Merriwether *et al., Journal of Molecular Evolution* 33:543-555, 1991.

In addition to pharmacologic agents, abnormal mitochondrial function in diabetic patients can be corrected by transfer of normal mitochondrial genes into the nucleus of a patient's cells, particularly cells which are subject to diabetic complications and/or already compromised by diabetic complications. An expression cassette is prepared which includes an amino-terminal mitochondrial targeting sequence and an appropriate promoter together with the first exon and upstream sequences of a nuclear-encoded oxidative phosphorylation gene (for example, ATP synthase) and the mtDNA gene. For synthesis of this construct, *see*, for example, Nagley *et al., PNAS* 85:2091-5, 1988; and Gray *et al., Methods in Enzymology* 264:369-89, 1996. Appropriate promoters capable of directing the transcription of a cloned gene or cDNA include both viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus (CMV) promoter (Boshart *et al*, (1985) *Cell* 41:521-530), the SV40 promoter (Subramani *et al*, (1981) *Mol. Cell. Biol.* 1:854-864) and the major late promoter from Adenovirus 2 (Kaufman and Sharp, (1982) *Mol. Cell. Biol.* 2:1304-13199). Promoters for tissue specific expression *in vivo* include the following. For cardiac compartment-specific expression, the alpha-myosin heavy chain promoter can be used to obtain transgene expression in adult atria and ventricles (Colbert *et al*, (1997) *Clin. Invest.* 100:1958-1968) and Masalci *et al*, (1998) 101:527-535). Neuron specific expression can be

obtained using the neuron-specific enolase (NSE) promoter (Klein *et al.*, (1998) *Exp. Neurol.* 150:183-194 and Rondi-Reig *et al.*, (1997) *Neuroreport* 8:2429-2432).

The expression cassette can be inserted into a viral vector with the appropriate tissue tropism. Examples of viral vectors include adenovirus, *Herpes simplex* virus, adeno-associated virus, retrovirus, lentivirus, and the like. See Blomer *et al.*, *Human Molecular Genetics* 5 Spec No:1397-404, 1996; and Robbins *et al.*, *Trends in Biotechnology* 16:35-40, 1998. Adenovirus-mediated gene therapy has been used successfully to transiently correct the chloride transport defect in nasal epithelia of patients with cystic fibrosis. See Zabner *et al.*, *Cell* 75:207-216, 1993. In addition to providing a method for mitochondrial gene therapy for patients in need thereof, the invention provides for a method of selecting non-mutated mitochondria by transfecting a cell with peptide-nucleic acids having sequences complimentary to mitochondrial DNA that contain a deletion breakpoint or single base mutation as compared to wild-type mitochondrial DNA so that replication of the mitochondrial DNA is blocked and mitochondria with DNA mutations are eliminated.

As an alternative to correction of abnormal mitochondrial function is elimination of mitochondria with ROS-promoting mutations from heteroplasmic diabetic cells by selectively inhibiting the replication of the mutant mtDNA. This can be done using published techniques of synthesizing peptide nucleic acids complementary to human mtDNA templates containing a deletion breakpoint or single base mutation. See Taylor *et al.*, *Nature Genetics* 15:212-215, 1997. "Complementary" nucleic acid refers to a nucleic acid sequence which selectively hybridizes to a nucleic acid probe. For discussion of nucleic acid probe design and annealing conditions see for example Sambrook *et al.*, *Molecular Cloning: A laboratory Manual* (2<sup>nd</sup> ed.), Vols 1-3 Cold Spring Harbor Laboratory (1989) or *Current Protocols in Molecular Biology*, F. Ausubel *et al.*, Ed Greene Publishing and Wiley-Interscience, New York (1987) each of which is incorporated herein by reference. Techniques for manipulation of nucleic acids are described generally in Sambrook, *supra*.

In the gene therapy methods of the invention, transfection *in vivo* is obtained by introducing a therapeutic transcription or expression vector into the mammalian host, either as naked DNA, complexed to lipid carriers, particularly cationic lipid carriers, or inserted into a viral vector, for example a recombinant adenovirus. The introduction into the mammalian host can be by any of several routes, including intravenous or intraperitoneal injection, intratracheally, intrathecally, parenterally, intraarticularly, intranasally, intramuscularly,

topically, transdermally, application to any mucous membrane surface, corneal installation, etc. Of particular interest is the introduction of a therapeutic expression vector into a circulating bodily fluid or into a body orifice or cavity, such as the heart. Thus, intravenous administration and intrathecal administration are of particular interest since the vector may be widely disseminated following such routes of administration, and aerosol administration finds use with introduction into a body orifice or cavity. Particular cells and tissues can be targeted, depending upon the route of administration and the site of administration. For example, a tissue which is closest to the site of injection in the direction of blood flow can be transfected in the absence of any specific targeting. An arterial catheter can be used to introduce the expression vector into an organ such as the heart or kidney. The eye can be accessed directly either by the use of ocular drops or by injecting into the eye. For accessing nerves, this can be by injection into the nerve or injection into the region of the cell body. If lipid carriers are used, they can be modified to direct the complexes to particular types of cells using site-directing molecules. Thus, antibodies or ligands for particular receptors or other cell surface proteins may be employed, with a target cell associated with a particular surface protein. An amino terminal mitochondrial targeting sequence joined to a nucleic acid can be used to target the nucleic acid to the mitochondria. See Taylor *et al*, *Nature Genetics* 15:212-215, 1997.

Any physiologically acceptable medium may be employed for administering the DNA, recombinant viral vectors or lipid carriers, such as deionized water, saline, phosphate-buffered saline, 5% dextrose in water, and the like as described above for the pharmaceutical composition, depending upon the route of administration. Other components may be included in the formulation such as buffers, stabilizers, biocides, etc. These components have found extensive exemplification in the literature and need not be described in particular here. Any diluent or components of diluents that would cause aggregation of the complexes should be avoided, including high salt, chelating agents, and the like.

The amount of therapeutic vector used will be an amount sufficient to provide for a therapeutic level of expression in a target tissue susceptible to diabetic complications or for adequate dissemination to a variety of tissues after entry into the bloodstream and to provide for a therapeutic level of expression in susceptible target tissues. A therapeutic level of expression is a sufficient amount of expression to prevent, treat, or palliate one or more diabetic complication or the symptoms of diabetic complications. In addition, the dose of the

nucleic acid vector used must be sufficient to produce a desired level of transgene expression in the affected tissue or tissues *in vivo*. Other DNA sequences, such as adenovirus VA genes can be included in the administration medium and be co-transfected with the gene of interest. The presence of genes coding for the adenovirus VA gene product may significantly enhance the translation of mRNA transcribed from the expression cassette if this is desired.

A number of factors can affect the amount of expression in transfected tissue and thus can be used to modify the level of expression to fit a particular purpose. Where a high level of expression is desired, all factors can be optimized, where less expression is desired, one or more parameters can be altered so that the desired level of expression is attained. For example, if high expression would exceed the therapeutic window, then less than optimum conditions can be used.

The level and tissues of expression of the recombinant gene may be determined at the mRNA level as described above, and/or at the level of polypeptide or protein. Gene product may be quantitated by measuring its biological activity in tissues. For example, protein activity can be measured by immunoassay as described above, by biological assay such as inhibition of ROS, or by identifying the gene product in transfected cells by immunostaining techniques such as probing with an antibody which specifically recognizes the gene product or a reporter gene product present in the expression cassette.

Typically, the therapeutic cassette is not integrated into the patient's genome. If necessary, the treatment can be repeated on an *ad hoc* basis depending upon the results achieved. If the treatment is repeated, the patient can be monitored to ensure that there is no adverse immune or other response to the treatment.

The compositions comprising agents identified as directly or indirectly inhibiting ROS in insulin independent cells, the mtDNA and polynucleotide compositions of the present invention are useful in treatment and prevention of complications related to hyperglycemia and diabetes. They also can be used to treat other diseases in which mtDNA is damaged due to elevated intracellular ROS. These diseases include Alzheimer's Disease, Parkinson's Disease, and other age-related, tissue-degenerative diseases, as well as arterogenic effects of leptin in patients with impaired glucose tolerance and obese non-diabetic patients.

Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Pharmaceutically acceptable carriers and formulations for use in the present invention are found in Remington's *Pharmaceutical Sciences*, Mack Publishing Company,

Philadelphia, PA, 17th ed. (1985), which is incorporated herein by reference. For a brief review of methods for drug delivery, see Langer, (1990) *Science* 249:1527-1533, which is incorporated herein by reference.

In preparing pharmaceutical compositions of the present invention, it may be desirable to modify the compositions of the present invention to alter their pharmacokinetics and biodistribution. For a general discussion of pharmacokinetics, see Remington's *Pharmaceutical Sciences*, *supra*, Chapters 37-39. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art (See, e.g., Langer, *supra*). Examples of such methods include protection of the agents in vesicles composed of substances such as proteins, lipids (for example, liposomes), carbohydrates, or synthetic polymers. For example, the agents of the present invention can be incorporated into liposomes in order to enhance their pharmacokinetics and biodistribution characteristics. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka *et al*, (1980) *Ann. Rev. Biophys. Bioeng.* 9:467, U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028, all of which are incorporated herein by reference.

The agents of the present invention can be used in pharmaceutical compositions that are useful for administration to humans. The pharmaceutical compositions of the invention are intended for parenteral, topical, oral or local administration. For example, the pharmaceutical compositions can be administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. The invention provides compositions that comprise a solution of the agents described above dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of pharmaceutically acceptable aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions can be sterilized by conventional, well known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions can contain as pharmaceutically acceptable carriers, substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like.



For solid compositions, conventional nontoxic pharmaceutically acceptable carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient and more preferably at a concentration of 25%-75%.

For aerosol administration, the pharmaceutical compositions are preferably supplied in finely divided form along with a surfactant and propellant as pharmaceutically acceptable carriers. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides, can be employed. A carrier also can be included, as desired, as with, for example, lecithin for intranasal delivery.

The pharmaceutical compositions of the invention can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, and capsules. The effective amount of a particular agent in a pharmaceutical composition depends on, for example, the chemical nature of the agent, the manner of administration, the weight and general state of health of the patient, the severity of the disease being treated and the judgment of the prescribing physician. Dosages, formulations and administration schedules can vary in these patients as compared to normal individuals. In general, dosages range from about 100  $\mu$ g to about 500 mg or more, with dosages of from about 250  $\mu$ g to about 50 mg being more commonly used. It must be kept in mind that the materials of the present invention may be employed in serious disease or injury states, and in such cases it is possible and may be felt desirable by the treating physician to administer substantial excesses of these compositions.

The effect of treatment of diabetic complications can be evaluated as follows. Where it is desired to treat a particular diabetic complication, both the biological efficacy of the treatment modality as well as the clinical efficacy are evaluated, if possible. For example, in the treatment of diabetic cardiomyopathy, which manifests itself by subclinical abnormalities of left-ventricular diastolic function, such as impaired left-ventricular relaxation on digitized

M-mode or Doppler echocardiography, the biological efficacy of the treatment can be evaluated, for example, by observation of return of the impaired left-ventricular relaxation to normal or cessation of progression. The clinical efficacy, whether treatment of the underlying defect is effective in changing the course of disease, can be more difficult to measure. While the evaluation of the biological efficacy goes a long way as a surrogate endpoint for the clinical efficacy, it is not definitive. Thus, measuring a clinical endpoint which can give an indication of left-ventricular diastolic function after, for example, a six-month period of time, can give an indication of the clinical efficacy of the treatment regimen.

Similarly, in the treatment of another diabetic complication, diabetic retinopathy, therapy can be evaluated by before and after treatment observations of changes in the disease manifestations. Diabetic retinopathy is divided into three levels—(1) no diabetic retinopathy, (2) nonproliferative diabetic retinopathy, and (3) proliferative diabetic retinopathy. In addition, macular edema can be present at any level of retinopathy and is also graded. No retinopathy refers to a clinically normal fundus. Nonproliferative retinopathy is subdivided into four groups: mild, moderate, severe and very severe. Each level is precisely defined by the presence and degree of specific clinical features—hemorrhages with or without microaneurysms, venous bleeding, and intraretinal microvascular abnormalities (*see* Early Treatment Diabetic Retinopathy Study Report Number 10: Grading diabetic retinopathy from stereoscopic color fundus photographs—an extension of the modified Airlie House classification. *Ophthalmology* 98:786-806, 1991). Proliferative retinopathy indicates the presence of any retinal neovascularization. The extent and progression or regression of diabetic retinopathy are evaluated and monitored by fundus photographs, direct or indirect ophthalmoscopy, and fluorescein angiography of the retina. The presence of retinal thickening within the macula constitutes macular edema. This is assessed by slit-lamp microscopy. In terms of clinical features of diabetic retinopathy, even extensive proliferative changes may cause no visual symptoms until vitreous hemorrhage or retinal detachment occurs. Retinal detachment not observable due to vitreous hemorrhage can be detected by B-scan ultrasonography. Macular edema causes a non-correctable decline in visual acuity. Absence of progression or regression of these changes after a period in which they generally would be expected to develop in a patient with a particular level of diabetic retinopathy can give an indication of the clinical efficacy of the treatment regimen. Similarly, one skilled in the art can evaluate the biological and clinical efficacy of a particular gene therapy protocol

for other diseases mediated by ROS-induced mutations in mtDNA. The manifestations and clinical features of other diabetic complications are described in Brownlee and King, *Endocrinology and Metabolism Clinics of North America*, 25:1-483, 1996.

The subject compositions can be provided for use in one or more procedures. For treatment with a pharmaceutical composition comprising an agent identified as one which is effective in directly or indirectly inhibiting ROS production in insulin independent cells, the subject compositions can be provided as kits for use in one or more operations. The kits will include a composition comprising an effective agent either as concentrates (including lyophilized compositions), which may be further diluted prior to use or they may be provided at the concentration of use, where the vials may include one or more dosages. Kits for genetic therapy usually will include the therapeutic mtDNA construct either as naked mtDNA with or without mitochondrial targeting sequence peptides, as a recombinant viral vector or complexed to lipid carriers. Additionally, lipid carriers and mitochondrial targeting peptides can be provided in separate containers for complexing with the provided mtDNA. The therapeutic mtDNA can be present as concentrates which may be further diluted prior to use or they may be provided at the concentration of use, where the vials may include one or more dosages. Conveniently, in the kits single dosages can be provided in sterile vials so that the physician may employ the vials directly, where the vials will have the desired amount and concentration of agents. When the vials contain the formulation for direct use, usually there will be no need for other reagents for use with the method. The subject compositions can be contained in packaging material, which comprises a label indicating that the subject compositions can be used to treat diabetic complications in humans.

The following examples are offered by way of illustration and not by way of limitation.

## **EXAMPLES**

### **Example 1**

#### **Effect of different mitochondrial blockades on intracellular reactive oxygen species.**

Bovine aorta endothelial (BAE) cells were obtained from the N.I.A. cell repository in Camden, N.J.. The cells were cultured in Eagles Minimal Essential Medium (MEM) containing 10% fetal bovine serum (FBS). Replicate cultures of  $6-8 \times 10^6$  cells in 300 mm dishes were prepared. The experimental medium is MEM plus 0.4% FBS to put the cells in a

growth-arrested state. Cytosolic NADH is transferred into mitochondria predominantly through the malate-aspartate shuttle, while mitochondrial NADH and FADH<sub>2</sub> are generated by the TCA cycle from cytosolic pyruvate. To determine the role of the malate-aspartate shuttle in hyperglycemia-induced ROS production, bovine aortic endothelial cells were incubated in the presence of aminooxyacetate, a well-characterized inhibitor of the malate-aspartate shuttle.

The intracellular formation of reactive oxygen species was detected by using the fluorescent probe CM-H<sub>2</sub>DCFDA (Molecular Probes Inc., Eugene, OR). Cells (5 x 10<sup>5</sup>/ml) were loaded with 10μM CM-H<sub>2</sub>DCFDA, incubated for 45min at 37°C, and analyzed in a HTS 7000 Bio Assay Fluorescent Plate Reader (Perkin Elmer) using the HTSoft program. No effect on hyperglycemia-induced ROS production was observed (Figure 1). In contrast, inhibition of glycolysis-derived pyruvate transport into mitochondria by 4-hydroxycyanocinnamic acid completely inhibited hyperglycemia-induced ROS production (Figure 1). Flux through the TCA cycle was measured by <sup>14</sup>CO<sub>2</sub> production from [U-<sup>14</sup>C] glucose (Ishihara *et al*, *Diabetes* (1996) 45:1238-1244. Cells were preincubated for 30 min in HBKRBB containing 5mM or 30mM glucose. 500 μl of HBKRBB containing 1μCi/ml of U-<sup>14</sup>C glucose was added, and tubes were placed in a vial containing 500 μl of Hyamine hydroxide. After 2h incubation at 37°C, 100 μl of 4N sulfuric acid was injected into each tube and vials were incubated overnight at room temperature. Tubes were removed and the hyamine hydroxide was counted in 10 ml of Aquasol 2. Cellular protein content was determined by Coomassie Plus (Pierce) after solubilization in 0.1N NaOH. Flux through the TCA cycle was increased 2.2-fold by 30mM glucose (0.183 ± 0.005 nmol/mg/min vs. 0.084 ± 0.005 nmol/mg/min for 5mM glucose). These data indicate that the TCA cycle is the source of increased ROS-generating substrate induced by hyperglycemia.

To determine the site of hyperglycemia-induced intracellular reactive oxygen species (ROS) production, BAE were incubated with either amytal (500μM), an inhibitor of Complex I, TTFA (10μM), an inhibitor of Complex II, carbonyl cyanide m-chlorophenylhydrazone (CCCP) (0.5μM), an uncoupler of oxidative phosphorylation that abolishes the mitochondrial membrane proton gradient, 4-OHCA (250μM), or AOAC (100μM). Compared with baseline conditions (5mM glucose), incubation with 30mM glucose increased ROS production from 59.61 ± 6.78 (5mM glucose) to 150 ± 8.00 μmol/ml (Figure 1). Amytal did not reduce increased ROS production, while both TTFA and CCCP completely prevented the effect of hyperglycemia (Figure 1). These data show that ROS

generation arises exclusively from Complex II activity, presumably through the ubisemiquinone radical-generating Q cycle (Ingledew and Ohnishi, *Biochem J.* (1977) 164:617-620).

### Example 2

#### Effect of different mitochondrial blockades on cellular lipid peroxidation.

BAE cells were obtained as described in Example 1. Lipid peroxides were determined fluorimetrically (Giardino *et al*, *J. Clin. Invest.* (1996) 97:1422-28). Figure 2 shows levels of lipid peroxidation as a percentage of the value of cells incubated in low glucose determined as TBARS in BAE cells incubated for 168 hours in medium containing either 5 mM glucose, 30 mM glucose or 30 mM glucose plus 2mM azide, 10  $\mu$ M TTFA or 0.5  $\mu$ M CCCP. Lipid peroxide (LP) levels in cells grown in media containing 30mM glucose and TTFA are approximately equivalent to LP levels in cells grown in media containing only 5mM glucose. LP levels in cells grown in media containing 30mM glucose and CCCP are decreased as compared to cells grown in 30mM glucose alone to approximately 120% of LP levels in cells grown in media containing only 5mM glucose; cells grown in 30mM glucose have approximately 150% the LP levels seen in cells grown in 5mM glucose. Likewise, for cells grown in 30mM glucose plus CCCP, the levels of LP are decreased as compared to cells grown in 30mM glucose alone to approximately the level in cells grown in 5mM glucose. Cells grown in 30mM glucose and azide have LP levels higher than for cells grown in 30mM glucose alone (approximately 180% of LP levels measured in cells grown in 5mM glucose). Lipid peroxides were determined fluorimetrically as described in Giardino *et al*, *J. Clin. Invest.* (1996) 97:422-28.

### Example 3

Hyperglycemia activates NF $\kappa$ B, in part by activation of PKC (Pieper and Haq, *J. Cardiovasc. Pharmacol* (1997) 30:528-532.) The effect of TTFA and CCCP on NF $\kappa$ B activation therefore were evaluated. BAE cells were obtained as described in Example 1. Figure 3A shows levels of NF $\kappa$ B activation (percent of low glucose) as measured using an electrophoretic mobility shift assay kit (Gel Shift Assay Kit) from Promega (Madison, WI); the kit was used according to the manufacturer's instructions. Gels were transferred to Whatman paper, dried and placed in film holders with XAR-5 films at -80°C. Films were

scanned using an Ultrascan XL densitometer (Pharmacia). BAE cells incubated for 2 hours in medium containing either 5 mM glucose, 30 mM glucose or 30 mM glucose plus 2mM azide, 0.5  $\mu$ M oligomycin, 10  $\mu$ M TTFA or 0.5  $\mu$ M CCCP. NF $\kappa$ B activation levels in cells grown in media containing 30mM glucose and TTFA or CCCP are reduced to a level approximately equivalent to NF $\kappa$ B activation levels in cells grown in media containing only 5mM glucose, whereas cells grown in 30mM glucose alone have nearly 200% the NF $\kappa$ B activation levels seen in cells grown in 5mM glucose. Cells grown in 30mM glucose and azide or oligomycin have NF $\kappa$ B activation levels that are increased above the levels for cells grown in 30mM glucose alone to between approximately 250-280% of NF $\kappa$ B activation levels measured in cells grown in 5mM glucose. This activation by glucose was specific for the NF $\kappa$ B p50 subunit (lower band of NF $\kappa$ B binding activity, Figure 3B). Again, NF $\kappa$ B activation by 30mM glucose was completely inhibited by both TTFA and CCCP. This observation indicates that glucose activates endothelial cell NF $\kappa$ B by inducing mitochondrial ROS overproduction, rather than by inducing activation of ROS-producing cytosolic NADPH oxidases.

#### Example 4

##### Effect of different mitochondrial blockades on levels of methylglyoxal-derived AGEs.

Excessive production of AGEs also appears to play a major role in the pathogenesis of diabetic complications (*Brownlee Annu. Rev. Med.* (1995) 46:223-34. Some AGEs require oxygen for their formation, while the generation of others is thought to be oxygen-independent (Baynes *et al*, *Diabetes* (1996) 48:1-9).] BAE cells were obtained as described in Example 1.

Figure 4 shows levels of methylglyoxal-derived AGEs (MAGEs) as determined by scanning densitometry (Maytin *et al*, *Anal Biochem* 194: 284-294, 1991) of immunoblots made with a 1:4000 dilution of 1H7G5 antibody (Giardino *et al*, *Diabetes* 47 (Supp 1) A:123, 1998) in BAE cells incubated for 168 hours in medium containing either 5 mM glucose, 30 mM glucose or 30 mM glucose plus 2mM azide, 10  $\mu$ M TTFA or 0.5  $\mu$ M CCCP. Cells grown in 30mM glucose have approximately 2 times the MAGE levels seen in cells grown in 5mM glucose. Cells grown in 30mM glucose and azide have MAGE levels approximately 2.5 times those of cells grown in 5mM glucose. MAGE levels in cells grown in media containing 30mM glucose and TTFA or CCCP are equivalent to MAGE levels in cells grown in media containing only 5mM glucose. Since methylglyoxal is formed by fragmentation of glyceraldehyde-3-phosphate, this dependency on increased mitochondrial ROS production may

reflect the well-described reversible inhibition of glyceraldehyde-3-phosphate dehydrogenase by ROS, which increases glyceraldehyde-3-phosphate levels (Knight *et al*, *Cardiovasc. Res.* (1996) 32:1016-1023).

### Example 5

#### Effect of different mitochondrial blockades on intracellular glyoxal and/or fructosyllysine-derived AGE formation.

The effect of TTFA and CCCP on hyperglycemia-induced formation of GFAGE also was examined. BAE cells were obtained as described in Example 1. Figure 5 shows levels of glyoxal and/or fructosyllysine-derived AGEs (GFAGEs) as determined by scanning densitometry (Maytin *et al.*, *Analytical Biochem* (1991) 194:284-294) of immunoblots made with 6D12 antibody (Ikeda *et al*, *Biochem* 35:8075-83, 1996) in BAE cells incubated for 168 hours in medium containing either 5 mM glucose, 30 mM glucose or 30 mM glucose plus 10  $\mu$ M TTFA or 0.5  $\mu$ M CCCP. GFAGE levels in cells grown in media containing 30mM glucose and TTFA or CCCP are reduced to approximately equivalent to GFAGE levels in cells grown in media containing only 5mM glucose as compared to cells grown in 30mM glucose alone which have approximately twice the GFAGE levels seen in cells grown in 5mM glucose. Cells grown in 30mM glucose and azide have GFAGE levels approximately 2.5 times of those measured in cells grown in 5mM glucose.

### Example 6

#### Effect of different mitochondrial blockades on PKC activity.

Activation of PKC appears to play a major role in the pathogenesis of diabetic complications (Kaya and King, *Diabetes* (1998) 47:859-866). Therefore, the effect of TTFA and CCCP on hyperglycemia-induced activation of PKC was evaluated. BAE cells were obtained as in Example 1. Figure 6 shows PKC activity (pmol/min/ mg protein) in BAE cells incubated for 5 days in medium containing either 5 mM glucose, 30 mM glucose or 30 mM glucose plus either 10  $\mu$ M TTFA or 0.5  $\mu$ M CCCP. Subcellular fractions were prepared and PKC activities in soluble and detergent-solubilized preparations were determined as the phosphorylation of acetylated myelin basic protein amino acids 4-14 ((Ac-MBP) 4-14). For the soluble fraction (cytosol), there was no difference in the PKC activity among the cells incubated in high glucose with or without TTFA and CCCP as compared to low glucose.

However, in the detergent solubilized (membrane) fraction, there was a significant ( $p < 0.01$ ) difference in the PKC activity in cells incubated in high glucose as compared to low glucose. The PKC activity in the presence of either TTFA or CCCP reduced the PKC activity to a level comparable to that of cells incubated in low glucose, suggesting that mitochondrial ROS overproduction initiates the hyperglycemia-induced *de novo* synthesis of diacylglycerol or phosphatidylcholine hydrolysis that activate PKC (Koya and King, *Diabetes* (1998) 47:859-866). TTFA did not inhibit PKC activity *in vitro* (data not shown).

### Example 7

#### Effect of electron transport Complex II inhibition on hyperglycemia-induced sorbitol accumulation.

Increased flux through the polyol pathway is a third mechanism that appears to play a major role in the pathogenesis of diabetic complications (Lee *et al*, *Proc. Nat'l Sci USA* (1995) 92:2780-4). In this pathway, elevated glucose concentration results in increased production of sorbitol by the enzyme aldose reductase. BAE cells obtained as described in Example 1 were incubated for 240 h. 30 $\mu$ l of a neutralized perchloric acid cell extract was added to 100 $\mu$ l of working reagent containing 23 Units Diaphorase, 40 mg NAD and 50  $\mu$ l resazurin/50 ml triethanolamine buffer pH 8.5. 0.15U of sorbitol dehydrogenase in 10  $\mu$ l was then added. The assay was done in a 96 well fluorescence plate and incubated at room temperature with an opaque cover and mixed on an orbital shaker for 60 minutes. Fluorescence was determined at excitation 560 emission 580 using a Perkin Elmer model LS50 B fluorimeter (Malone *et al*, *Diabetes* (1980) 29:861-864). Sorbitol levels were 4-fold higher than baseline (5mM glucose) when endothelial cells were incubated in 30mM glucose. Sorbitol accumulation at both glucose concentrations was completely inhibited by the specific aldose reductase inhibitor zopolrestat, indicating that in bovine aortic endothelial cells, sorbitol is derived exclusively from aldose reductase activity (data not shown). Hyperglycemia-induced sorbitol accumulation was reduced 75% by TTFA (10 $\mu$ M) (Figure 7), indicating that mitochondrial ROS-overproduction stimulates aldose reductase activity. This observation is consistent with recent data suggesting that aldose reductase activity is reversibly down-regulated by nitric oxide modification of a cysteine residue in the enzyme's active site (Chandra *et al*, *Biochim Biophys Acta* (1997) 1341:217-222), and that ROS appear to reduce



nitric oxide levels in diabetic endothelium (Pieper and Haq, *J. Cardiovas. Pharmacol.* (1997) 30:528-532). TTFA did not inhibit aldose reductase activity *in vitro* (data not shown).

### Example 8

#### Effect of electron transport Complex II inhibition on tyrosine kinase vascular endothelial growth factor receptor.

In order to test whether inhibition of mitochondrial ROS overproduction would reverse a diabetes-induced abnormality for which the mechanism is currently undefined, expression of the tyrosine kinase vascular endothelial growth factor receptor flk-1 was examined. Flk-1 mRNA and protein are both increased in retinae of diabetic rats (Hammes *et al. Diabetes* (1998) 47:401-6). In BAE, 30mM glucose increased flk-1 protein levels by 2-fold compared to levels at 5mM glucose ( $637 \pm 52$  vs.  $329 \pm 523$  AU). Equal amounts of cell extract protein were used for quantitative immunoblotting (Giardino *et al., J. Clin. Invest.* (1996) 97:1422-28). Flk-1 was detected using a polyclonal antibody (0.1 $\mu$ g/ml) obtained from Dr. Georg Breier, Max-Planck - Institute, Bad Nauheim, Germany. TTFA (10 $\mu$ M) completely inhibited 30mM glucose-induced flk-1 expression ( $223 \pm 52$  AU). These results are shown in Figure 8.

### Example 9

#### Effect of superoxide dismutase/catalase mimetics on hyperglycemia-induced ROS.

The purpose of this experiment was to determine whether a superoxide dismutase/catalase mimetic could decrease accumulation of hyperglycemia-induced reactive oxygen species. BAE cells were obtained as described in Example 1. Figure 9 shows the results of incubating the cells for 24 hours in medium containing either 5mM glucose, 30mM glucose, or 30mM glucose plus 10nM Mn (111) tetrakis (benzoic acid) porphyrin (MnTBAP), 25nM TBAP, 50nM TBAP, 100nM TBAP, 150nM TBAP, or 200nM TBAP. Intracellular reactive oxygen species were measured as described in Example 1. At a concentration of 50nM or higher TABP, inhibition of hyperglycemia-induced ROS is complete.

### Example 10

#### Effect of mitochondrial inhibitors on hyperglycemia-induced ROS levels in rat hepatocytes.

The purpose of this experiment was to determine whether agents which inhibited  
5 accumulation of hyperglycemia-induced ROS accumulation in aortic endothelial cells (*see*  
Example 1) would also inhibit accumulation of hyperglycemia-induced ROS in hepatocytes.

Rat hepatocytes obtained from Dr. David Neufeld, Liver Research Center, Albert  
Einstein College of Medicine, were incubated 24 hours in a 96-well plate (200,000 cells/well)  
in 5mM glucose alone, and in 30mM glucose, alone, and with either aminooxyacetate  
10 (AOAC) (100 $\mu$ M), 4-hydroxycyanocinnamic acid (4-OHCA) (250 $\mu$ M), amytal (500 $\mu$ M),  
thenoyltrifluoroacetone (TTFA) (10 $\mu$ M), carbonyl cyanide m-chlorophenylhydrazone (CCCP)  
(0.5 $\mu$ M), or MnTBAP (100 $\mu$ M), and ROS were quantited using the fluorescent probe CM-  
H2DCFDA as described under Example 1. As with endothelial cells, high glucose induced a  
4-fold increase in ROS. Inhibition of NADH transport into mitochondria by AOAC had no  
15 effect, while inhibition of pyruvate transport with 4-OHCA completely inhibited  
hyperglycemia-induced ROS. Inhibition of Complex I by amytal had no effect, while  
inhibition of Complex II by TTFA completely inhibited hyperglycemia-induced ROS.  
Uncoupling of oxidative phosphorylation with CCCP had an identical effect. The superoxide  
dismutase/catalase mimetic MnTBAP also completely normalized the hyperglycemia-induced  
20 ROS. These results are shown in Figure 10.

### Example 11

#### The effects of TTFA or MnTBAP on intracellular lipoprotein modification in human hepatocytes.

The purpose of this experiment is to evaluate whether inhibitors of mitochondrial  
25 electron transport complexes or superoxide dismutase/catalase mimetics can be used to modify  
intracellular lipoproteins. Cultured human hepatocytes are prepared and maintained as  
described by Davis (Davis, *Methods in Enzymology* (1986) 129:227-283). Cultured  
hepatocytes are incubated in 5mM glucose, 30mM glucose, and 30mM glucose plus an  
30 inhibitor of a mitochondrial electron transport complex, such as TTFA (10 $\mu$ M), or a  
superoxide dismutase/catalase mimetic, such as MnTBAP (50-100 $\mu$ M). Culture medium  
obtained from these incubations is subjected to lipoprotein separation by ultracentrifugation

(Macfarlane *et al.*, *Electrophoresis* (1997) 18:1796-806). The VLDL, HDL, and LDL fractions are then analyzed for oxidatively modified and AGE-modified lipoproteins using capillary electrophoresis (Stocks and Miller, *Journal of Lipid Research* (1998) 39:1305-9), gas chromatography-mass spectrometry analysis of extracted lipids (Al-Abed *et al.*, *J. Biol. Chem.* (1996) 271:2892-6) and enzyme-linked immunosorbant assays using antibodies to oxidation products, such as 4-hydroxynonenal (Waeg *et al.*, *Free Radical Research* (1996) 25:149-59) and AGEs (Bucala *et al.*, *Proc. Natl. Acad. Sci.* (1994) 91:9441-5).

### Example 12

#### The effects of TTFA and MnTBAP on differential expression of complication-promoting genes.

The purpose of this experiment is to determine the effects of TTFA or MnTBAP on abnormal hepatocyte gene expression of glucose-6-phosphatase, apolipoproteins, and other complication-promoting genes. Total RNA is isolated and quantitative competitive RT-PCR is performed using the Access RT-PCR system (Promega). The competitor RNA is constructed using the RT-PCR Competitor Construction Kit (Ambion), which generates an RNase-resistant competitor. Glucose-6-phosphate protein levels are assessed using Western blots with an anti-glucose-6-phosphate antibody according to the method of Lange *et al.* (*J. Biol. Chem.* 261:101-107, 1986). Similar methods are used to assess the effects of TTFA or MnTBAP on abnormal hepatocyte gene expression of apolipoproteins and other proteins influencing the atherogenic process negatively or positively. Probes are designed for doing quantitative RT-PCR from appropriate genebank sequences, and antibodies for Western blotting are available from commercial sources. Cells are incubated in 5mM glucose, 30mM glucose, or 30mM glucose plus either an inhibitor of a mitochondrial electron complex, such as TTFA (10μM) or a superoxide dismutase/catalase mimetic, such as MnTBAP (50-100μM), as described in Example 10.

### Example 13

#### Inhibition of leptin induced increases in ROS

The purpose of this experiment was to evaluate the effect of inhibitors of ROS production on leptin induced increases in ROS. Bovine aortic cells as described in Example 1

were preincubated with TTFA or CCCP or carrier for an hour then incubated with or without leptin for 45 minutes at room temperature and measured with DCF method as in Example 1. The intracellular formation of reactive oxygen species was detected by using the fluorescent probe CM-H<sub>2</sub>DCFDA (Molecular Probes Inc., Eugene, OR). Cells (5 x 10<sup>5</sup>/ml) were loaded with 10μM CM-H<sub>2</sub>DCFDDA, incubated for 45min at 37°C, and analyzed in a HTS 7000 Bio Assay Fluorescent Plate Reader (Perkin Elmer) using the HTSoft program. Data represent the mean +/- SE for 5 experiments. As shown in Figure 11, leptin induced ROS increases in bovine endothelial cells were prevented by either (a) inhibiting mitochondrial electron transport complex II with TTFA, or (b) partially uncoupling mitochondrial electron transport with CCCP in the presence of non-diabetic glucose levels (5mM).

#### Example 14

##### The effect of mitochondrial inhibitors on hyperglycemia-induced increases in glucose-6 phosphatase gene expression

Rat hepatocytes were incubated under standard conditions (Seoane, J., *et al.*, *J. Biol. Chemistry* 272:26972-26977, 1977) with either 5mM glucose, 30mM glucose, or 30 mM glucose plus TTFA, CCCP, or MnTBAP, as described in Example 10. After 24 hours, mRNA was prepared and quantitative RT-PCR performed. Data was normalized for beta actin amplified in the same tubes. The results are shown in Figure 12: mitochondrial inhibitors inhibited hyperglycemia-induced increases in glucose-6 phosphatase gene expression. The Y-axis shows the densitometric readings which correspond to relative glucose-6 phosphatase mRNA levels. Data represent the mean +/- SE for 3 experiments.

#### Example 15

##### The effect of mitochondrial inhibitors on hyperglycemia-induced increases in hepatic glucose production

Rat hepatocytes were incubated under standard conditions (Seoane, J., *et al.*, *J. Biol. Chemistry* 272:26972-26977, 1977) with either 5mM glucose, 30mM glucose, or 30mM glucose plus TTFA, CCCP, or MnTBAP, as described in Example 10. After 24 hours, hepatic glucose output (primarily gluconeogenesis) was determined as described in conditions

(Seoane, J., *et al.*, *J. Biol Chemistry* 272:26972-26977, 1977). Briefly, Cells were incubated in the presence of gluconeogenic substrates (5mM alanine, 5mM glycine, 5mM glutamine, 10mM lactate, 1mM pyruvate) in the absence of glucose for 2 hours. Media were collected for glucose determination by the standard glucose oxidase method. The results are shown in Figure 13: mitochondrial inhibitors inhibited hyperglycemia-induced increases in hepatic glucose production. Data represent the mean +/- SE for 9 experiments.

The above results demonstrate hyperglycemia-induced elevation of mitochondrial ROS, elevation of lipid peroxides, activation of NFκB, elevation of both methylglyoxal-derived and glyoxal/fructosyllysine-derived advanced glycosylation endproducts (AGEs), activation of PKC, increased aldose reductase activity, and increased Flk-1 expression. Elevated levels of lipid peroxides, AGEs, Flk-1 and activation of NFκB, PKC, and aldose reductase are correlated with ROS levels. The increased ROS is substantially normalized by the oxidative phosphorylation uncoupler *m*-chlorophenylhydrazine (CCCP), the Complex II inhibitor theonyltrifluoroacetone (TTFA), and the superoxide dismutase mimetic, MnTBAP. In contrast, azide, an inhibitor of mitochondrial electron transport Complex IV, is shown to aggravate the high glucose effect in all examples, while a Complex V inhibitor oligomycin is shown to aggravate the high glucose effect in the example where ROS are directly measured, and the example where NFκB activation is measured.

All publications and patent applications mentioned in this specification are indicative of the level of skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now having been fully described, it will be apparent to one ordinarily skilled in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method for screening a plurality of pharmaceutical agents to identify one or more member of said plurality that is effective in decreasing accumulation of hyperglycemia-induced reactive oxygen species under high glucose conditions, said method comprising:  
5 measuring the level of reactive oxygen species produced in a cell culture system of insulin-independent cells grown under high glucose conditions in the presence of individual members of said plurality of agents, whereby one or more member of said plurality of agents that is effective is identified by a decrease in the level of reactive oxygen species formed as  
10 compared to the level of reactive oxygen species formed in a cell culture system of insulin-independent cells grown under high glucose conditions in the absence of a member of said plurality of agents.

2. The method according to Claim 1, wherein said insulin-independent cells are endothelial cells.

15 3. The method according to Claim 2, wherein said endothelial cells are aorta endothelial cells.

4. The method according to Claim 3, wherein said aorta endothelial cells are bovine aorta endothelial cells.

20 5. The method according to Claim 1, wherein said accumulation of hyperglycemia-induced reactive oxygen species is inhibited by a method selected from the group consisting of partially uncoupling oxidative phosphorylation from electron transport in mitochondria, inhibiting a mitochondrial electron transport complex which is a site of reactive oxygen species generation in said insulin-independent cell, dismutating at least one of superoxide and hydrogen peroxide, and inhibiting binding and activation of hexokinase  
25 isoforms to or by the mitochondrial membrane.

6. The method according to Claim 1, wherein said level of reactive oxygen species produced is decreased by preventing formation of said reactive oxygen species.

7. A method for screening a plurality of agents to identify one or more member of said plurality of agents that is effective in inhibiting a mitochondrial electron transport complex under high glucose conditions, said method comprising:  
30

measuring the level of reactive oxygen species produced in a cell culture system of insulin-independent cells in high glucose in the presence of individual members of said

plurality of agents, whereby one or more member of said plurality of agents that is effective in inhibiting said mitochondrial electron transport complex which is a site of ROS generation in said insulin-independent cell is identified by a decrease in the level of reactive oxygen species formed as compared to the level of reactive oxygen species formed in a cell culture system of insulin-independent cells in high glucose in the absence of a member of said plurality of agents.

8. The method according to Claim 7, wherein said insulin-independent cells are aortic endothelial cells or hepatocytes and said mitochondrial electron transport complex is Complex II.

9. A method for screening a plurality of agents to identify one or more member of said plurality of agents that is effective in dismutating at least one of superoxide and hydrogen peroxide under high glucose conditions, said method comprising:

measuring the level of reactive oxygen species produced in a cell culture system of insulin-independent cells in high glucose in the presence of individual members of said plurality of agents, whereby one or more member of said plurality of agents that is effective in dismutating at least one of superoxide and hydrogen peroxide is identified by a decrease in the level of reactive oxygen species formed as compared to the level of reactive oxygen species formed in a cell culture system of insulin-independent cells in high glucose in the absence of a member of said plurality of agents.

10. A method for reducing production of reactive oxygen species in an insulin-independent blood element exposed to high glucose conditions, said method comprising:

providing said blood element with a composition comprising a sufficient amount of an agent which inhibits production of hyperglycemia-induced reactive oxygen species in said insulin-independent blood element by a method selected from the group consisting of partially uncoupling oxidative phosphorylation from electron transport in mitochondria; inhibiting a mitochondrial electron transport complex which is a site of reactive oxygen species generation in said insulin-independent blood element; dismutating at least one of superoxide and hydrogen peroxide; and, inhibiting binding and activation of hexokinase isoforms to or by the mitochondrial membrane.

11. The method according to Claim 10, wherein said mitochondrial electron transport complex is Complex I or Complex II.

12. The method according to Claim 11, wherein said agent is carbonyl cyanide *m*-chlorophenylhydrazone, theonyltrifluoroacetone, amytal, idebenone, or manganese tetrakis (benzoic acid) porphyrin.

13. The method according to Claim 10, wherein said insulin-independent blood element is a cell involved in at least one of vascular disease and neurological disease.

14. The method according to Claim 13, wherein said disease is dysfunction.

15. The method according to Claim 10, wherein said insulin-independent blood element is selected from the group consisting of a vascular cell, a peripheral neuron, a circulating blood element, and an hepatocyte.

16. The method according to Claim 15, wherein said circulating blood element is selected from the group consisting of a platelet, a monocyte, a macrophage, a lymphocyte, and an hepatocyte.

17. The method according to Claim 10, wherein said production of hyperglycemia-induced reactive oxygen species is by normal mitochondria in said insulin-independent blood element.

18. An article of manufacture comprising packaging material and a composition comprising a pharmaceutical agent which decrease the level of hyperglycemia-induced ROS in insulin-independent cells contained within said packaging material, whereas said composition is effective for treating diabetic complications, and wherein said packaging material comprises a label which indicates that said composition is approved for human use.

19. A method for decreasing accumulation of reactive oxygen species in an insulin-independent cell exposed to high glucose conditions, said method comprising:

providing said cell with a composition comprising a sufficient amount of a superoxide dismutase/catalase mimetic to decrease accumulation of hyperglycemia-induced reactive oxygen species in said insulin-independent cell.

20. A method for inhibiting glucose-induced activation of a cellular process in an insulin-independent cell, said method comprising:

providing said cell with a composition comprising a sufficient amount of an agent which decreases accumulation of reactive oxygen species, whereby a cellular process selected from the group consisting of PKC activation, AGE formation, polyol/sorbitol pathway activity, glucosamine pathway activity, and NF $\kappa$ B is inhibited.

21. The method according to Claim 20, wherein said agent is selected from the



group consisting of carbonyl cyanide *m*-chlorophenylhydrazone, theonyltrifluoroacetone, and manganese tetrakis (benzoic acid) porphyrin.

22. A method for inhibiting a cellular pathway, said method comprising:

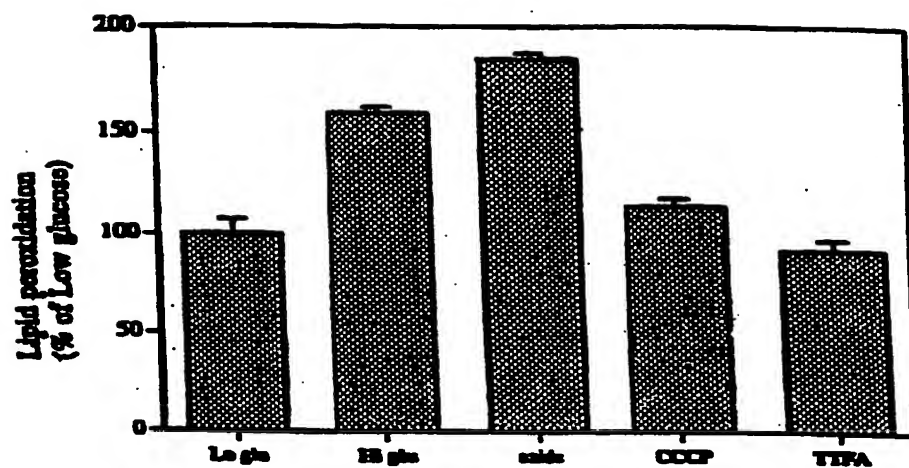
providing a cell with a composition comprising a sufficient amount of an agent which  
5 decreases accumulation of mitochondrially derived reactive oxygen species in said cell  
selected from the group consisting of carbonyl cyanide *m*-chlorophenylhydrazone, theonyltrifluoroacetone, and manganese tetrakis (benzoic acid) porphyrin, whereby a cellular  
pathway selected from the group consisting of PKC activation, AGE formation,  
polyol/sorbitol pathway activity, glucosamine pathway activity, and NFκB is inhibited.



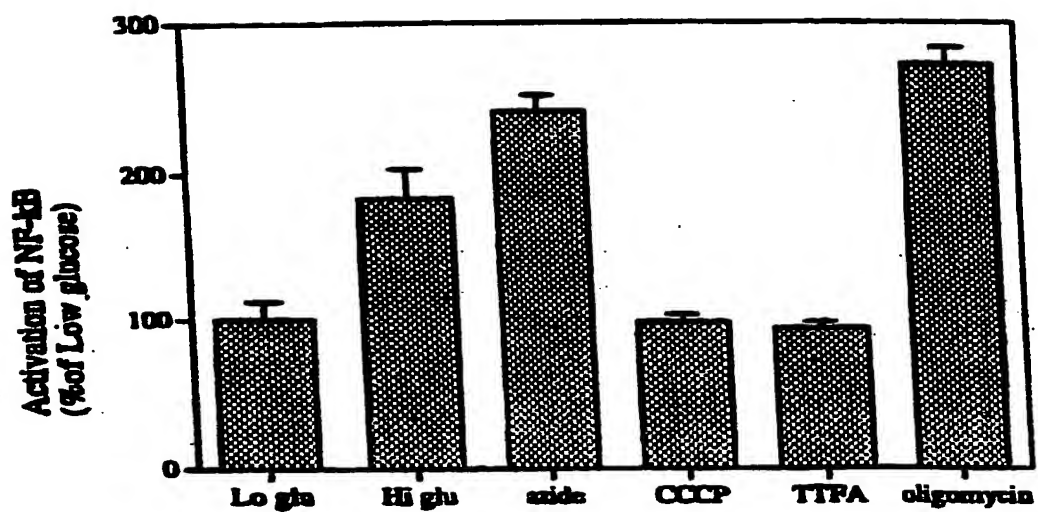
FIGURE 1

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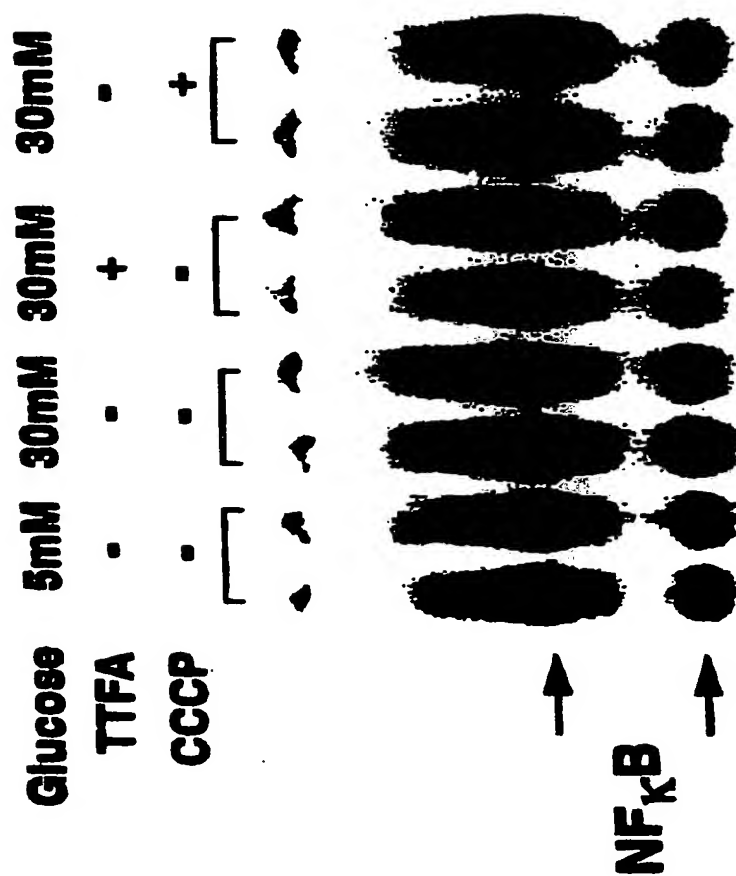
**FIGURE 2**

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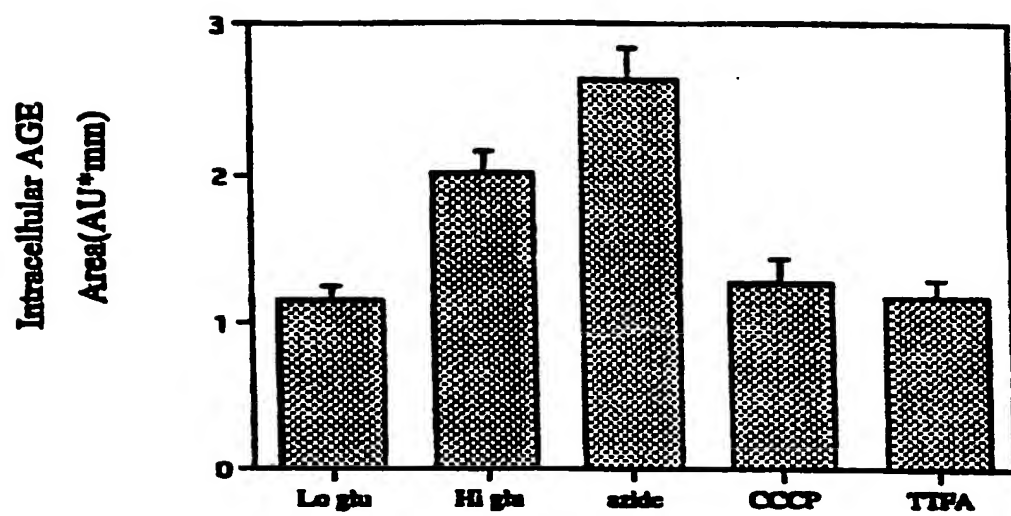
**FIGURE 3A**

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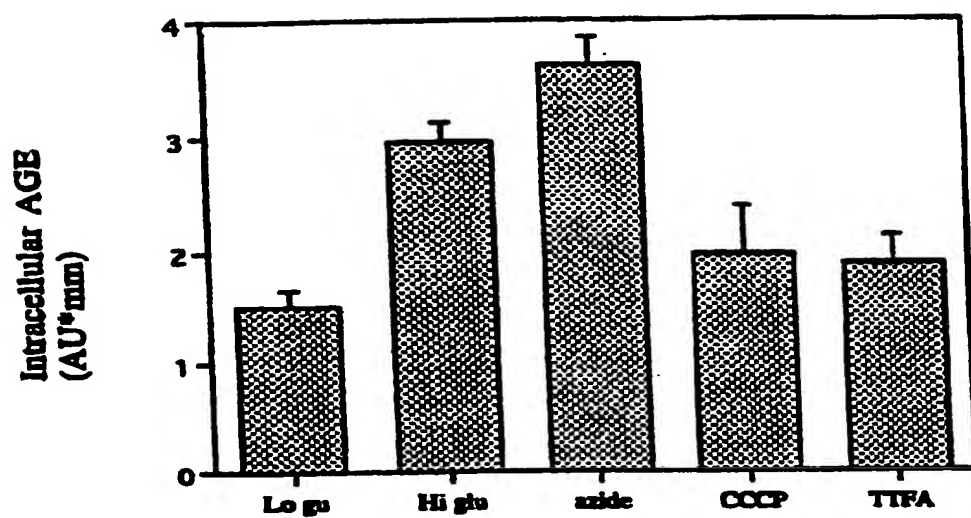
FIGURE 3B



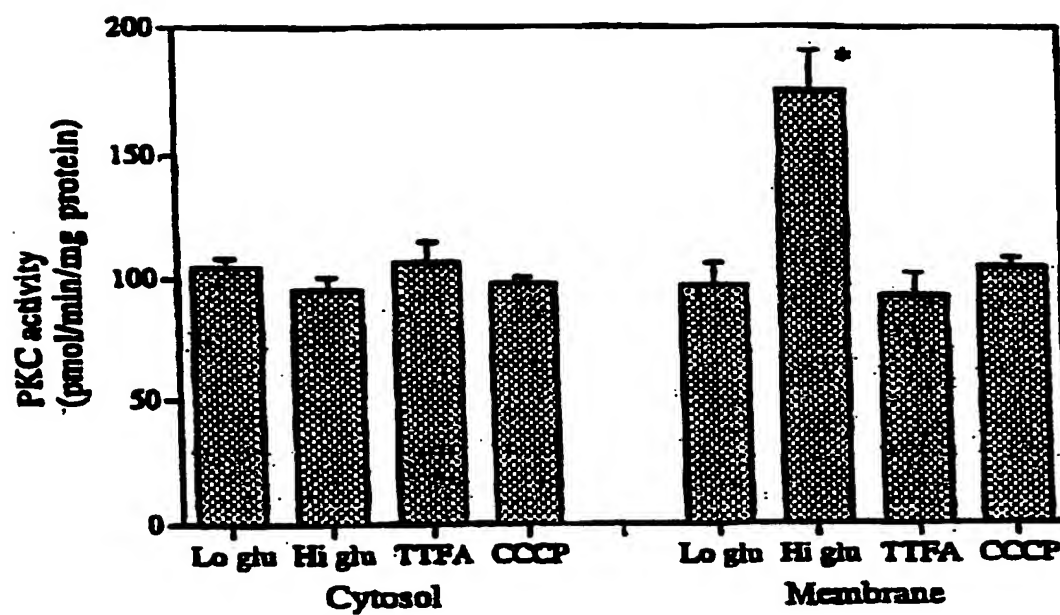
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**FIGURE 4**

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**FIGURE 5**

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**FIGURE 6**



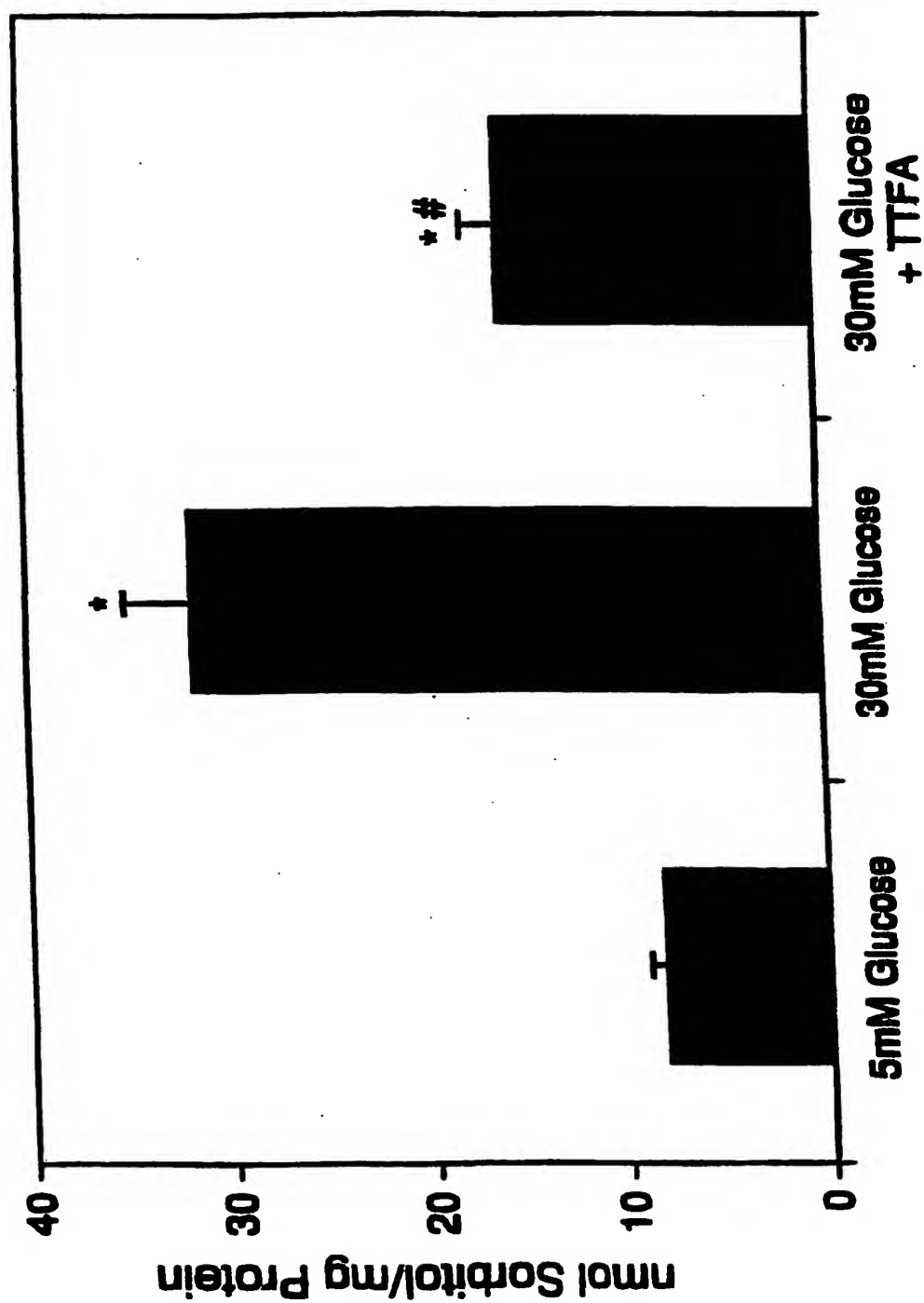


FIGURE 7

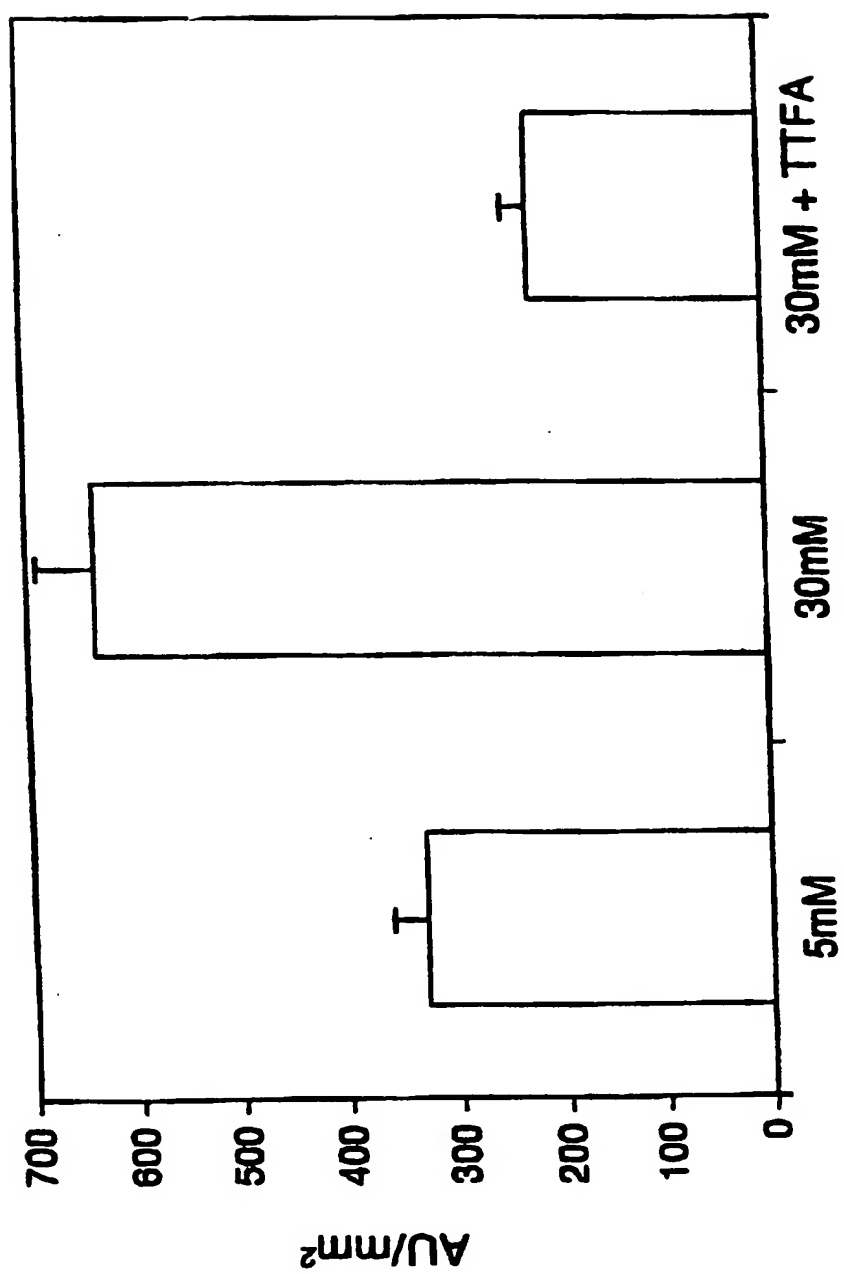


FIGURE 8

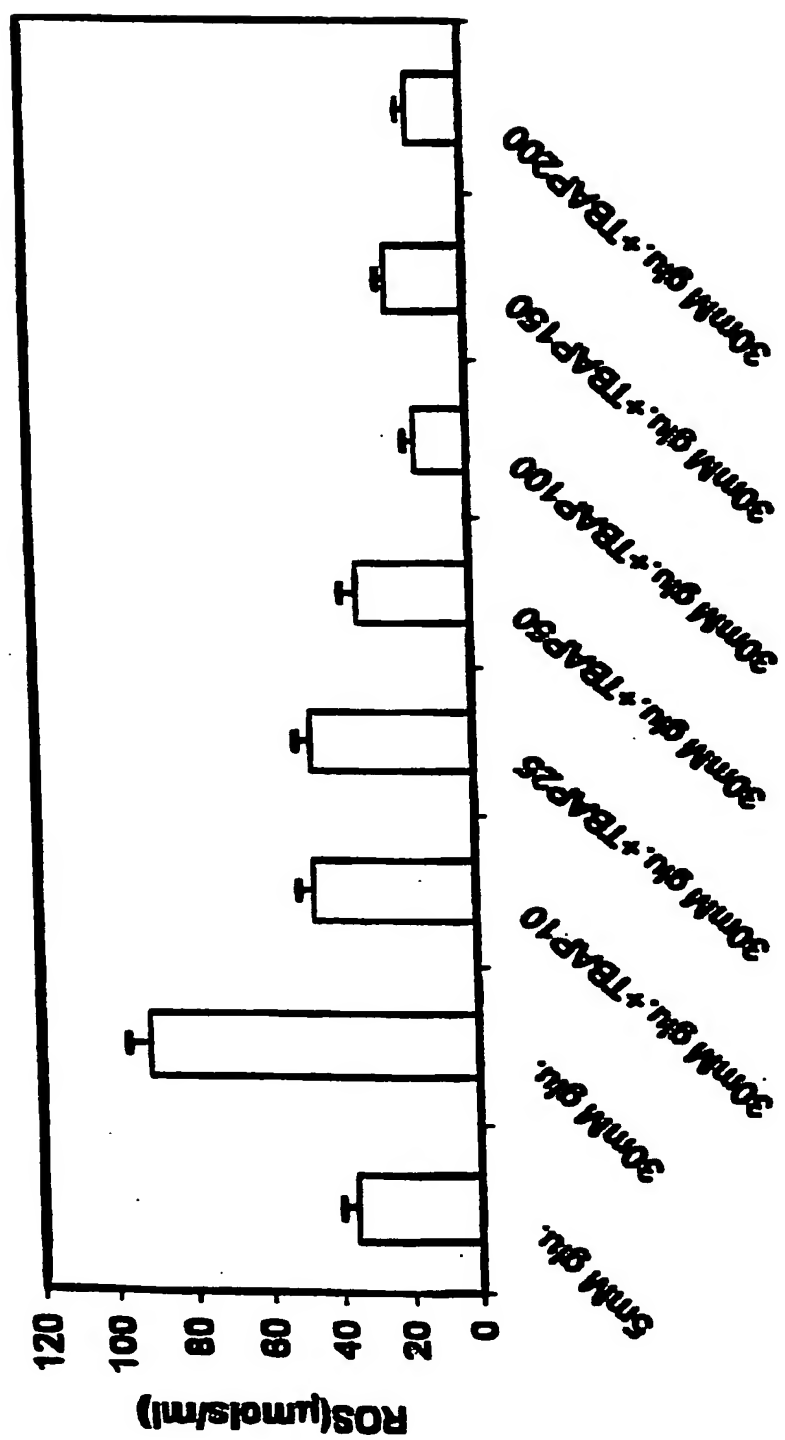


FIGURE 9

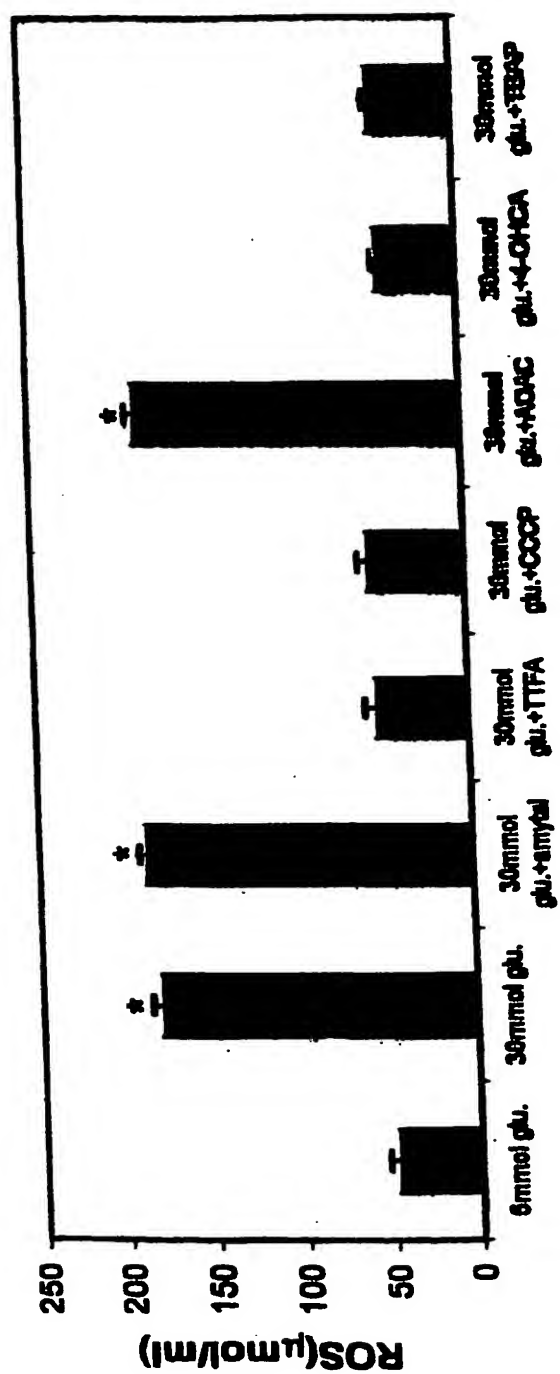
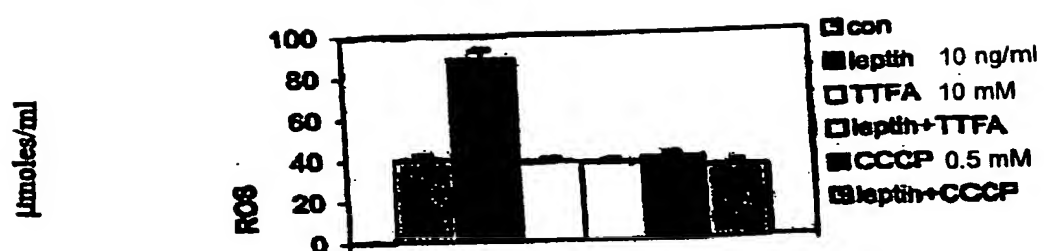
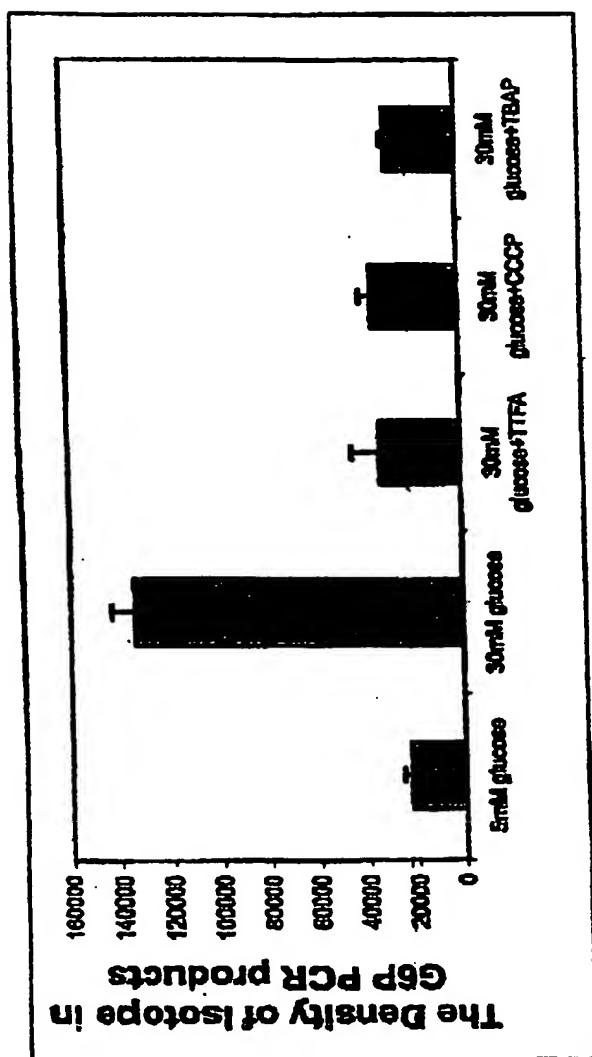


FIGURE 10

**FIGURE 1 1**

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**FIGURE 12**

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The effect of ROS inhibitors on the glucose production in rat hepatocytes

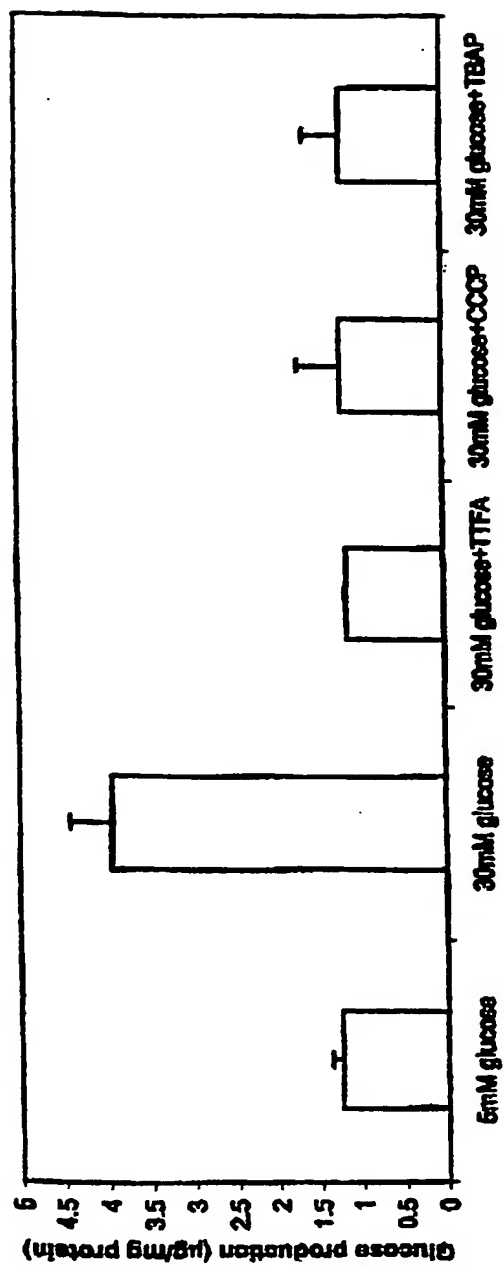


FIGURE 13

(19) World Intellectual Property Organization  
International Bureau



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(10) International Publication Number  
**WO 00/19993 A3**

(51) International Patent Classification<sup>7</sup>: G01N 33/50,  
A61K 31/12, 31/15, 31/40, 31/515, 31/52, A61P 3/10

(72) Inventor: BROWNLEE, Michael; 1300 Morris Park Avenue, F-531, Bronx, NY 10461 (US).

(21) International Application Number: PCT/US99/23457

(74) Agent: RAE-VENTER, Barbara; Rae-Venter Law Group, P.C., P.O. Box 60039, Palo Alto, 94306 (US).

(22) International Filing Date: 6 October 1999 (06.10.1999)

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MEDICINE OF YESHIVA UNIVERSITY [US/US]; a  
division of Yeshiva University, 1300 Morris Park Avenue,  
Bronx, NY 10461 (US).

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: METHODS AND COMPOSITIONS FOR DECREASING MITOCHONDRIAL OVERPRODUCTION OF REACTIVE OXYGEN SPECIES IN CELLS

(57) Abstract: The subject invention provides means of identifying agents identified as capable of reducing levels of ROS or ROS level correlated indicators such as levels of LPs, AGEs, NFkB, PKC, or aldose reductase activation in insulin-independent cells grown under high glucose conditions.

WO 00/19993 A3



# INTERNATIONAL SEARCH REPORT

Inter. Appl. No.

PCT/US 99/23457

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/50 A61K31/12 A61K31/15 A61K31/40 A61K31/515  
A61K31/52 A61P3/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, EMBASE, EPO-Internal, WPI Data, PAJ, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KING, G.L. AND BROWNLE, M.: "The cellular and molecular mechanisms of diabetic complications" ENDOCRINOLOGY AND METABOLISM CLINICS OF NORTH AMERICA, vol. 25, no. 2, June 1996 (1996-06), pages 255-270, XP000878790 the whole document page 263, last line -page 264, paragraph 1 ---	1-9
X	WO 98 27426 A (UNIV ABERDEEN ;ASHFORD MICHAEL LAWRENCE JAMES (GB); HERSON PACO SA) 25 June 1998 (1998-06-25) claim 1 --- -/-	1-9



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

19 September 2000

Date of mailing of the international search report

14.11.00

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# INTERNATIONAL SEARCH REPORT

Inter. Application No

PCT/US 99/23457

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CURCIO, F. AND CERIELLO, A.: "Decreased cultured endothelial cell proliferation in high glucose medium is reversed by antioxidants: new insights on the pathophysiological mechanisms of diabetic vascular complications." IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY, vol. 28A, no. 11-12, November 1992 (1992-11) - December 1992 (1992-12), pages 787-790, XP000879178 abstract	1-9
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A	DATABASE WPI Section Ch, Week 199308 Derwent Publications Ltd., London, GB; Class B05, AN 1993-061598'08! XP002131312 "New drug for preventing and treating diabetic complications comprises 1,4-benzoquinone or its hydroquinone form; treats nephropathy, neuropathy, arteriosclerosis etc." & JP 05 009114 A (TAKEDA CHEM IND LTD), 19 January 1993 (1993-01-19) abstract	1-9
X		10-20

-/-

# INTERNATIONAL SEARCH REPORT

Inter. Application No

PCT/US 99/23457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WACHSMAN JOSEPH T: "The beneficial effects of dietary restriction: Reduced oxidative damage and enhanced apoptosis." MUTATION RESEARCH, vol. 350, no. 1, 1996, pages 25-34, XP000900797  ISSN: 0027-5107  abstract  page 26, column 1, paragraph 1 -page 28, column 2, paragraph 3  page 31, column 2, paragraph 4 -page 32, column 1, paragraph 1</p>	10-22
X	<p>HONG, C. Y. ET AL: "The inhibitory effect of some ionophores on human sperm motility" CONTRACEPTION (1986), 33(3), 301-6 , XP000900566  abstract  page 302, paragraph 2</p>	18
X	<p>QUILLET-MARY, ANNE ET AL: "Implication of mitochondrial hydrogen peroxide generation in ceramide-induced apoptosis" J. BIOL. CHEM. (1997), 272(34), 21388-21395 , XP000900626  abstract</p>	10-12, 15-22
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X	<p>HAWTIN S.R. ET AL: ".beta.-amyloid inhibition of MTT reduction is not mimicked by inhibitors of mitochondrial respiration." BIOCHEMICAL SOCIETY TRANSACTIONS, (1995) 23/1 (56S). , XP000900574  the whole document</p>	10-13, 17,18,22
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International Application No  
PCT/US 99/23457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	KOSOVSKII, M. I. ET AL: "In vitro sugar transport regulation by new calcium ionophores" TSITOLOGIYA (1984), 26(4), 409-14 , XP000900593 abstract	10-12, 17-22
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X	MIYAZAKI M ET AL: "MECHANISMS RESPONSIBLE FOR LONG-TERM SURVIVAL OF ADULT RAT HEPATOCYTES IN THE PRESENCE OF PHENOBARBITAL IN PRIMARY CULTURE" EXPERIMENTAL CELL RESEARCH, vol. 182, no. 2, 1989, pages 415-424, XP000937422 ISSN: 0014-4827 abstract page 420, paragraph 2 -page 422, paragraph 1; figure 5	10-12, 15-20
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Int. Application No

PCT/US 99/23457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	<p>MIASNIKOV L A ET AL: "The effect of certain pharmacologic substances on cholesterol metabolism in experimental atherosclerosis !. Vliianie neirotropnykh farmakologicheskikh veshchestv na kholesterinovyi obmen pri eksperimental'nykh aterosklerozakh." PATOLOGICHESKAIA FIZIOLOGIIA I EKSPERIMENTALNAIA TERAPIIA, (1967 NOV-DEC) 11 (6) 40-3. , XP000937407 abstract</p>	10-20
X	<p>CALVERT A ET AL: "A double-blind evaluation of diazepam and amylobarbitone in acute myocardial infarction." MEDICAL JOURNAL OF AUSTRALIA, (1974 OCT 26) 2 (17) 624-7. , XP000937406 abstract</p>	10-12, 17-20
X	<p>CHIERICI F ET AL: "Clinical results of the use of a new drug association in heart diseases!. Risultati clinici dell'impiego di una nuova associazione farmacologica nelle cardiopatie." CLINICA TERAPEUTICA, (1970 FEB 15) 52 (3) 219-57. , XP000937403 page 256 -page 257</p>	10-13, 15,17-20
X	<p>ALEMA G ET AL: "Preliminary experiments on the effects of the intracarotid introduction of sodium amytal in parkinsonian syndromes." BOLL SOC ITAL BIOL SPER, (1961 OCT 31) 37 1036-7. , XP000937402 the whole document</p>	10-13, 15,18-20
X	<p>PARK JONG-WAN ET AL: "Role of mitochondria in oxidative damage of post-ischemic reperfused hearts." KOREAN JOURNAL OF PHARMACOLOGY, vol. 32, no. 2, 1996, pages 201-209, XP000900795 ISSN: 0377-9459 abstract</p>	10-13, 15,17-20

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International Application No

PCT/US 99/23457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>AMBROSIO, GIUSEPPE ET AL: "Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow"</p> <p>J. BIOL. CHEM. (1993), 268(25), 18532-41 , XP000939340 abstract</p> <p>---</p>	10-13, 15,17-20
◦ X	<p>LUFT R. ET AL: "Mitochondrial medicine."</p> <p>JOURNAL OF INTERNAL MEDICINE, (1995) 238/5 (405-421). , XP000900669 abstract page 405, column 1, paragraph 1 -page 417, column 1, paragraph 1</p> <p>---</p>	10-20
X	<p>ODAWARA M ET AL: "The effect of idebenone on insulin secretion in elderly diabetes mellitus."</p> <p>AGE (CHESTER), vol. 18, no. 3, 1995, page 140 XP000937416 6th Congress of the International Association of Biomedical Gerontology;Makuhari, Japan; August 21-23, 1995 ISSN: 0161-9152 abstract</p> <p>---</p>	10-20
X	<p>INAGAKI T. ET AL: "Psychiatric symptoms in a patient with diabetes mellitus associated with point mutation in mitochondrial DNA."</p> <p>BIOLOGICAL PSYCHIATRY, (1997) 42/11 (1067-1069). , XP000900663 page 1067, column 1, paragraph 1 -page 1068, column 2, paragraph 3</p> <p>---</p>	10-20
X	<p>SUNO M ET AL: "INHIBITORY EFFECTS OF A NOVEL COMPOUND IDEBENONE CV-2619 ON FREE RADICAL GENERATION AND LIPID PEROXIDATION"</p> <p>JOURNAL OF THE TAKEDA RESEARCH LABORATORIES, vol. 44, no. 1-2, 1985, pages 30-33, XP000900657 ISSN: 0371-5167 abstract</p> <p>---</p> <p style="text-align: center;">-/--</p>	10-12, 15,17-20

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/23457

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SENIN U ET AL: "IDEBENONE IN SENILE DEMENTIA OF ALZHEIMER TYPE: A MULTICENTRE STUDY"</p> <p>ARCHIVES OF GERONTOLOGY AND GERIATRICS,NL,ELSEVIER, AMSTERDAM, vol. 15, no. 3, 1 November 1992 (1992-11-01), pages 249-260, XP000671985</p> <p>ISSN: 0167-4943</p> <p>abstract</p>	10-13, 17-20
X	<p>WEYER G ET AL: "EFFICACY AND SAFETY OF IDEBENONE IN THE LONG-TERM TREATMENT OF ALZHEIMER'S DISEASE: A DOUBLE-BLIND, PLACEBO CONTROLLED MULTICENTRE STUDY"</p> <p>HUMAN PSYCHOPHARMACOLOGY. CLINICAL AND EXPERIMENTAL,XX,JOHN WILEY &amp; SONS LTD, vol. 11, no. 1, 1996, pages 53-65, XP000671987</p> <p>ISSN: 0885-6222</p> <p>abstract</p>	10-13, 17-20
X	<p>DELANTY N ET AL: "REVIEW ARTICLE. OXIDATIVE INJURY IN THE NERVOUS SYSTEM"</p> <p>ACTA NEUROLOGICA SCANDINAVICA,DK,MUNKSGAARD, COPENHAGEN, vol. 98, no. 3, September 1998 (1998-09), pages 145-153, XP000866110</p> <p>ISSN: 0001-6314</p> <p>the whole document</p>	10-13, 15,17-20
X	<p>IKEJIRI Y ET AL: "Idebenone improves cerebral mitochondrial oxidative metabolism in a patient with MELAS."</p> <p>NEUROLOGY, vol. 47, no. 2, 1996, pages 583-585, XP000900659</p> <p>ISSN: 0028-3878</p> <p>abstract</p> <p>page 583, column 1, paragraph 1</p> <p>page 584, column 2, paragraphs 1,2</p>	10-15, 17-20
X	<p>SCHUETZ EKKEHARD ET AL: "Suppression of leukocyte-enhanced cold ischemia/reperfusion injury of liver endothelium with the benzoquinone antioxidant idebenone."</p> <p>CLINICAL BIOCHEMISTRY, vol. 30, no. 8, December 1997 (1997-12), pages 619-624, XP000900660</p> <p>ISSN: 0009-9120</p> <p>abstract</p>	10-20

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Int. l. Application No

PCT/US 99/23457

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	SHOFFNER J.M. ET AL: "Oxidative phosphorylation diseases and mitochondrial DNA mutations: Diagnosis and treatment." ANNUAL REVIEW OF NUTRITION, (1994) 14/- (535-568). , XP000900670 page 543, paragraph 4 -page 554, paragraph 2 page 557, paragraph 3 ---	10-20
X	NAPPI G ET AL: "LONG-TERM IDEBENONE TREATMENT OF VASCULAR AND DEGENERATIVE BRAIN DISORDERS OF THE ELDERLY" ARCHIVES OF GERONTOLOGY AND GERIATRICS,NL,ELSEVIER, AMSTERDAM, vol. 15, no. 3, 1 November 1992 (1992-11-01), pages 261-269, XP000671984 ISSN: 0167-4943 abstract ---	10-15, 17-20
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X	SHIVARAM K ET AL: "Idebenone inhibits taurochenodeoxycholic acid toxicity and reduces oxidant stress in isolated rat hepatocytes." GASTROENTEROLOGY, vol. 108, no. 4 SUPPL., 1995, page A1169 XP000937412 95th Annual Meeting of the American Gastroenterological Association and Digestive Disease Week;San Diego, California, USA; May 14-17, 1995 ISSN: 0016-5085 abstract --- -/-	10-12, 15-22



# INTERNATIONAL SEARCH REPORT

Inte. l. onal Application No

PCT/US 99/23457

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WIELAND E ET AL: "Oxygen radicals (or) mediate the effect of white blood cells (WBC) on cytochrome P-450-3A4 (CPY3A4)-activity and -mRNA expression in a pig liver cold storage/perfusion model." HEPATOLOGY, vol. 26, no. 4 PART 2, 1997, page 629A XP000937417 48th Annual Meeting of the American Association for the Study of Liver Diseases; Chicago, Illinois, USA; November 7-11, 1997 ISSN: 0270-9139 abstract	10-12, 15-20
X	NAGAOKA A ET AL: "INHIBITORY EFFECT OF IDEBENONE CV-2619 A NOVEL COMPOUND ON VASCULAR LESIONS IN HYPERTENSIVE RATS" JAPANESE JOURNAL OF PHARMACOLOGY, vol. 36, no. 3, 1984, pages 291-300, XP000937975 ISSN: 0021-5198 abstract	10-13, 15-20
X	NAITO M. ET AL: "Protective effects of idebenone on vascular endothelial cells against toxicity induced by oxidatively modified low density lipoprotein." ARTERY, (1994) 20/6 (314-323). , XP000900673 abstract	10-13, 15,17-20
X	NAGAI Y ET AL: "BRAIN DISTRIBUTION OF IDEBENONE CV-2619 AND ITS EFFECT ON LOCAL CEREBRAL GLUCOSE UTILIZATION IN RATS" FOLIA PHARMACOLOGICA JAPONICA, vol. 88, no. 2, 1986, pages 109-124, XP000900676 ISSN: 0015-5691 abstract; table 6	10-13, 15,17-20
X,P	WO 99 23097 A (UNIV DUKE) 14 May 1999 (1999-05-14) abstract page 2, line 29 -page 3, line 14 page 12, line 29 -page 17, line 12 page 47, line 4-6 page 54; example 9	10-22

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/23457

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>MELOV S ET AL: "Superoxide and mitochondria: Consequences and potential therapies."  ENVIRONMENTAL AND MOLECULAR MUTAGENESIS,  vol. 31, no. SUPPL. 29, 1998, page 23  XP000937971  29th Annual Meeting of the Environmental Mutagen Society; Anaheim, California, USA;  March 21-26, 1998  ISSN: 0893-6692  abstract</p> <p>---</p>	10-22
X	<p>MELOV S ET AL: "A NOVEL NEUROLOGICAL PHENOTYPE IN MICE LACKING MITOCHONDRIAL MANGANESE SUPEROXIDE DISMUTASE"  NATURE GENETICS, US, NEW YORK, NY,  vol. 18, no. 2,  18 February 1998 (1998-02-18), pages  159-163, XP000904849  ISSN: 1061-4036  abstract; figures 1,2; tables 1,2</p> <p>---</p>	10-22
X	<p>PARIS D ET AL: "ROLE OF PEROXYNITRITE IN THE VASOACTIVE AND CYTOTOXIC EFFECTS OF ALZHEIMER'S SS-AMYLOID1-40 PEPTIDE"  EXPERIMENTAL NEUROLOGY, US, SAN DIEGO, CA,  vol. 152, July 1998 (1998-07), pages  116-122, XP000905157  abstract</p> <p>---</p>	1-15, 17-22
X	<p>CUZZOCREA, SALVATORE ET AL: "Beneficial effects of Mn(III) tetrakis (4-benzoic acid) porphyrin ( MnTBAP ), a superoxide dismutase mimetic, n carrageenan-induced pleurisy"  FREE RADICAL BIOL. MED. (1998), VOLUME  DATE 1999, 26(1/2), 25-33 ,  XP000900575  abstract</p> <p>---</p>	10-12, 15-22
X	<p>MOK J.S.L. ET AL: "Inhibition of nitroergic neurotransmission in the bovine retractor penis muscle by an oxidant stress: Effects of superoxide dismutase mimetics."  BRITISH JOURNAL OF PHARMACOLOGY, (1998)  124/1 (111-118). ,  XP000900565  abstract</p> <p>---</p> <p style="text-align: center;">-/--</p>	10-15, 17-22

# INTERNATIONAL SEARCH REPORT

In ational Application No

PCT/US 99/23457

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATEL M.: "Inhibition of neuronal apoptosis by a metalloporphyrin superoxide dismutase mimic." JOURNAL OF NEUROCHEMISTRY, (1998) 71/3 (1068-1074). , XP000900559 abstract	10-15, 17-22
X	DAY, BRIAN J. ET AL: "Manganic porphyrins possess catalase activity and protect endothelial cells against hydrogen peroxide-mediated injury" ARCH. BIOCHEM. BIOPHYS. (1997), 347(2), 256-262 , XP000900554 abstract	10-13, 15,17-22
X	SZABO C. ET AL: "Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger." FEBS LETTERS, (1996) 381/1-2 (82-86). , XP000930008 abstract page 83, column 2, paragraph 3 -page 86, column 1, paragraph 1	10-12, 15-22
X	WO 96 09053 A (UNIV DUKE) 28 March 1996 (1996-03-28) abstract; claim 1; figure 1	10-12, 17-22
X	WO 96 40658 A (MONSANTO CO ;LENNON PATRICK J (US); HENKE SUSAN L (US); ASTON KARL) 19 December 1996 (1996-12-19) abstract page 25, line 27 -page 27, line 2	10,13-20
X	WO 95 10185 A (UNIV DUKE ;UNIV ALABAMA AT BIRMINGHAM RES (US)) 20 April 1995 (1995-04-20) abstract page 23 page 28	10-22

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/23457

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 10-17,19-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

As a result of the prior review under R. 40.2(e) PCT,  
no additional fees are to be refunded.

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☒ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-9

Methods for screening a plurality of pharmaceutical agents to identify agents effective in decreasing hyperglycemia induced reactive oxygen species in insulin independent cells.

2. Claims: 10-22 (partially)

Use of pharmaceutical agents effective in reducing hyperglycemia induced reactive oxygen species to reduce hyperglycemia induced reactive oxygen species in insulin-independent cells, wherein the agent is carbonyl cyanide m-chlorophenylhydrazine (CA-REG nr. 2792-22-5).

3. Claims: 10-22 (partially)

Use of pharmaceutical agents effective in reducing hyperglycemia induced reactive oxygen species to reduce hyperglycemia induced reactive oxygen species in insulin-independent cells, wherein the agent is theonylphenylhydrazine.

4. Claims: 10-19 (partially)

Use of pharmaceutical agents effective in reducing hyperglycemia induced reactive oxygen species to reduce hyperglycemia induced reactive oxygen species in insulin-independent cells, wherein the agent is amytal.

5. Claims: 10-19 (partially)

Use of pharmaceutical agents effective in reducing hyperglycemia induced reactive oxygen species to reduce hyperglycemia induced reactive oxygen species in insulin-independent cells, wherein the agent is idebenone.

6. Claims: 10-22 (partially)

Use of pharmaceutical agents effective in reducing hyperglycemia induced reactive oxygen species to reduce hyperglycemia induced reactive oxygen species in insulin-independent cells, wherein the agent is manganese tetrakis (benzoic acid) porphyrin.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.2

Present claims 10-22 relate to a compound/product/use/therapeutic application defined (inter alia)

by reference to the following parameter(s):

P1: uncoupling oxidative phosphorylation from electron transport in

P2: inhibiting a mitochondrial electron transport complex

(I or II),

P3: dismutating at least one of superoxide dismutase and hydrogen peroxide,

P4: inhibiting and activation of hexokinase isoforms to or by the mitochondrial membrane

P5: inhibiting a cellular pathway selected from the group consisting of PKC activation, AGE formation, polyol/sorbitol pathway activity, glucosamine pathway activity, and NF-kappaB inhibition.

The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible.

Present claims 10,11,13-21 relate to a compound/product/use/therapeutic application defined by reference to a desirable characteristic or property, namely an agent which inhibits production of hyperglycemia-induced reactive oxygen species/ decreases accumulation of mitochondrially derived reactive oxygen species.

The claims cover all compound/product/use/therapeutic application having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compound/product/use/therapeutic application. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound/product/use/therapeutic application by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search for the second to sixth invention has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds specifically mentioned in claims 12 and 22, in relation to the treatment of diabetes, diabetic neuropathy, and diabetic vascular disease with due regard to the description.

Regarding claims 10-22, the term "reducing production of reactive oxygen species" is not an univocal therapeutic application because the artisan skilled in the field of therapy can not enumerate exhaustively which disease is comprised within this term.

Regarding claim 12, the second compound has been read as thenoyltrifluoroacetone in place of theonyltrifluoroacetone, in accordance with page 7, line 6 of the description.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Regarding claim 14, it is not clear which dysfunction is meant.  
Regarding claim 15,16: hepatocytes are not normally regarded as circulating blood elements.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/US 99/23457

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